AWARD NUMBER: **W81XWH-16-2-0058**

TITLE: **Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments**

PRINCIPAL INVESTIGATOR: **Melissa McDiarmid, M.D.**

RECIPIENT: **University of Maryland**
Baltimore MD 21201

REPORT DATE: **October 2017**

TYPE OF REPORT: **Annual**

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution is unlimited.

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2017	Annual	30 Sep 2016 - 29 Sep 2017

4. TITLE AND SUBTITLE
Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-16-2- 0058

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Melissa McDiarmid. M.D.. MPH. Stella Hines M.D.. MSPH. Joanna Gaitens Ph.D..
Email: mmcdiarm@som.umaryland.edu; shines@som.umaryland.edu; jgaitens@som.umaryland.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of Maryland
660 Lexington Avenue
Baltimore, Maryland 21201

8. PERFORMING ORGANIZATION REPORT

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick. Marvland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT
The ‘signature’ wound of current and recent conflicts in both Iraq and Afghanistan is that incurred via contact with improvised explosive devices (IEDs) and other high kinetic energy weapons. Beyond the traumatic injury inflicted, health risks from wound contamination with toxic metals must be managed, even as risk from these contaminants is not fully known. To provide a scientific evidence base to refine the clinical management of these patients, a multidisciplinary approach using animal models and patient data will be used. A laboratory rat model system (Project 1) will provide bio-kinetic and toxicological data on a variety of military-relevant metals implanted in the rats. (Project 2) will identify biomarkers of early effect in tissues and body fluids of the implanted animals. Using an existing national VA Embedded Fragment Registry of such injured patients, (Project 3) will assess kidney injury --the presumed target of toxic metal exposure-- and (Project 4) will assess pulmonary injury in these Veterans from both systemic metal absorption and presumed blast-induced -baro-trauma at the time of injury.

15. SUBJECT TERMS
Embedded metal fragments, health effects, military-relevant metals, laboratory rat, toxic metals, , registry, exposure

16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT	18. NUMBER	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
transcriptome	U	U	UU	106	

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

TABLE OF CONTENTS

1. **INTRODUCTION:** Narrative that briefly (one paragraph) describes the subject, purpose and scope of the research.

The ‘signature’ wound of current and recent conflicts in both Iraq and Afghanistan is that incurred via contact with improvised explosive devices (IEDs) and other high kinetic energy weapons. Beyond the traumatic injury inflicted, health risks from wound contamination with toxic metals must be managed, even as risk from these contaminants is not fully known. To provide a scientific evidence base to refine the clinical management of these patients, a multidisciplinary approach using animal models and patient data will be used. A laboratory rat model system (Project 1) will provide bio-kinetic and toxicological data on a variety of military-relevant metals implanted in the rats. (Project 2) will identify biomarkers of early effect in tissues and body fluids of the implanted animals. Using an existing national VA Embedded Fragment Registry of such injured patients, (Project 3) will assess kidney injury --the presumed target of toxic metal exposure-- and (Project 4) will assess pulmonary injury in these Veterans from both systemic metal absorption and presumed blast-induced -baro-trauma at the time of injury.

2. **KEYWORDS:** Provide a brief list of keywords (limit to 20 words).

Embedded metal fragments, health effects, military-relevant metals, laboratory rat, toxic metals, transcriptome, registry, exposure

3. ACCOMPLISHMENTS:

What were the major goals of the project?

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Major Task 1
Experimental Preparation
Year 1/Month 1 to Year 1/Month 6, 100% completed.

Major Task 2
Animal Ordering and Pellet Implantation Surgeries
Year 1/Month 6 to Year 3/Month 8, 50% completed.

Major Task 3
Animal Health Assessments and Urine Collections
Year 1/Month 9 to Year 3/Month9, 35% completed.

Major Task 4*
Euthanasia and Tissue Collection; Transfer of Research Samples to University of Kentucky
Year 2/Month 8 to Year 3/Month 9, 20% completed.
*(See pg. 8)
****All Year 1 sub-tasks are complete**

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Major Task 1
Experimental Preparation
Year 1/Month 1 to Year 1/Month 12, 100% completed.
****All Year 1 sub-tasks are complete**

PROJECTS 3 & 4:

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

The Major Tasks for Year 1 are shared by Projects 3 and 4.

Major Task 1
<u>Questionnaire development</u>
Year 1/Month 1 to Year 1/Month 12, 100% completed.
Major Task 2
<u>Obtain regulatory approvals</u>
Year 1/Month 1 to Year 2/Month 1, 95% completed.

Major Task 3
<u>Recruitment and questionnaire administration</u>
Year 1/Month 1 to Year 4/Month 9, 5% completed.

Major Task 5
<u>Collection and analyses of urine specimens</u>
Year 1/Month 1 to Year 4/Month 7, 10% completed.

Major Task 6
<u>Collection analyses of PFT and IOS findings</u>
Year 1/Month 1 to Year 4/Month 6, 10% completed.

****All Year 1 sub-tasks are complete**

What was accomplished under these goals?

<u>John F. Kalinich, Ph.D., Principal Investigator, Project 1</u>
"Health Effects of Embedded Fragments of Military-Relevant Metals"

During Year 1 of this project, financial accounts were established, project personnel were hired and trained, and the required regulatory assurances and approvals, including IACUC, were obtained. A small cohort of training rats were purchased and implanted with metal pellets to hone our surgical skills. The rats were later humanely euthanized and tissue samples collected. Some samples were shipped to the University of Kentucky (Project 2: PI: Dr. Charlotte Peterson) for their preliminary testing and to assess that enough sample was being collected and the shipping method suitable. During this training period, our laboratory also developed and standardized a urine collection procedure that reduces the stress on the rats. Two manuscripts were submitted for publication on this procedure. Also in Year 1, our laboratory was extremely fortunate to be joined by Dr. Jessica Hoffman (Federal Government (GS) Employee) and Dr. William Danchanko (CDR, U.S. Navy). Their participation is of no cost to the project. Dr. Hoffman's expertise with rats and neurobiology will allow us to expand our efforts into understanding the effect of metals solubilized from the embedded fragments on the blood-brain barrier. CDR Danchanko's experience investigating the effect of embedded metals on bone health will also greatly enhance the utility of this study.

Prior to initiating implantation surgeries, we were informed that the vivarium at the Armed Forces Radiobiology Research Institute (AFRRI) would be undergoing an extensive 18-month renovation, commencing in January 2018. Although our animals will continue to be housed at AFRRI, the size of our housing area will be diminished. While this event does not change our overall statement of work, it did prompt us to revamp our surgery schedule and move the pellet implantation surgeries of some of the experimental groups to earlier in the project (the year 1 surgery schedule can be found in the Appendices). Thus, in this year, the 12-

month experimental groups have been implanted and are scheduled for euthanasia in the July/August 2018 timeframe. The 3-month experimental groups have also been implanted and have been euthanized and samples collected. Implantation and euthanasia of the 1-month experimental groups will occur in the last quarter of 2017 (project year 2/months 1-4). The 6-month experimental groups will be implanted in the second quarter of 2018 (Project Year 2/Months 4-5) with euthanasia scheduled in the last quarter of 2018 (Project Year 3/Months 1-2).

Implantation surgeries proceeded with no issues and no adverse health effects were observed in the 3- and 12-month groups as a result of the metal pellets. However, during euthanasia of the 3-month cohort, several interesting observations were made. First, although not completely unexpected, the nickel-implanted rats begin to develop tumors around the implanted pellet. Tissue changes, indicative of tumor development, were also observed around the implanted cobalt pellets. Surprisingly, the implanted copper pellets completely dissolved in the muscle leaving only some discoloration of the muscle tissue. The implanted iron pellets also have begun to dissolve leaving darkly stained tissue. Photographs of the pellets and implantation sites can be found in the Appendices.

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

During Year 1 of this project, financial accounts were established and project personnel were hired and trained. Dr. Kalinich (Project 1) group purchased a small cohort of training rats and implanted metal pellets to hone their surgical skills. The rats were later humanely euthanized and tissue samples collected with some samples shipped to the University of Kentucky (Project 2: PI: Dr. Charlotte Peterson) for preliminary testing and to assess that enough sample was being collected and the shipping method suitable. During this training period, our laboratory optimized the isolation of exosomes from rodent blood and urine; this was necessary to determine the minimal amount of sample (either blood or urine) required to isolate exosomes for miRNA profiling. These preliminary studies determined that we are able to successfully isolate exosomes from 0.5 mL of serum and 2 mL of urine. All procedures are now in place to receive experimental samples starting in Year 2 of the project.

PROJECTS 3 & 4:

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator Project 3
"Biomarker Assessment of Kidney Injury from Metal Exposure in Embedded Fragment Registry Veterans"

Stella Hines, M.D., MSPH, Project Leader/Principal Investigator Project 4
"Respiratory Health in a Cohort of Embedded Fragment Registry Veterans Exposed to Blasts and Metals"

Two different populations of Veterans will be selected from the VA Toxic Embedded Fragment Registry to either receive an invitation to complete a questionnaire (Study Population #1), or to participate in a clinical assessment visit (Study Population #2). During Year 1 of the project, a questionnaire entitled, "Self-Reported Health Effects in Veterans with Blast and Embedded Metal Fragment Injuries" was developed to capture Veterans' health history, fragment-

related symptom complaints and exposure circumstances to be mailed to Study Population #1-(Questionnaire Only). An online version of this questionnaire has also been successfully designed. Additionally, an expanded questionnaire entitled “Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments” was created for Study Population #2 of Projects 3 and 4 (the Clinical Assessment Group), which captures additional details needed to interpret metal concentrations and renal findings.

All IRB approvals were obtained, including those from VA Central IRB and VA Research Committees at all local participating sites, and we initiated our submission to DoD Human Research Protections Office in September (see “Projects 3 and 4 Regulatory Approval Schedule” in appendices). Recruitment materials, which include letters and recruitment telephone scripts, were created for the Clinical Assessment and the Questionnaire-Only Groups. A detailed spot urine collection protocol was designed, which the Baltimore staff demonstrated step-by-step during a videoconference with all VA recruitment sites in September. Lastly, protocols for Pulmonary Function Testing and Impulse Oscillometry Testing that include standardized output report templates were developed. This insures that all sites will report data the same way. All pulmonary function lab staff were trained on the testing protocol and performance of IOS at the participating VA sites.

What opportunities for training and professional development has the project provided?

Nothing to report.

How were the results disseminated to communities of interest?

Nothing to report.

What do you plan to do during the next reporting period to accomplish the goals?

<u>John F. Kalinich, Ph.D., Principal Investigator, Project 1</u>
“Health Effects of Embedded Fragments of Military-Relevant Metals”

During Year 2 of the project, the 4 remaining rats in the 3-month cohort (depleted uranium implanted) will be humanely euthanized and samples collected. Samples from all 3-month groups will be shipped to the University of Kentucky (Project 2) for analysis. Health assessment data for the 3-month rats will be compiled and statistically analyzed. Urine collection and health assessments will continue for the 12-month cohort until they are euthanized in the July/August 2018 timeframe. The rats in the 1-month experimental groups will be implanted and euthanized during Project Year 2. Health assessment data for the 1-month rats will also be compiled and statistically analyzed in Year 2. Finally, pellet implantation surgeries for the 6-month cohort are scheduled for the April/May 2018 timeframe.

<u>Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2</u>
“Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds”

During Year 2 of the project, we plan to receive samples from the 3-month group during the initial part of the year with 12-month samples received later in the year. Total RNA will be

isolated from 3-month skeletal muscle samples and prepared for microarray analysis. In addition, we plan to isolate exosomes from both blood and urine followed RNA isolation for miRNA profiling. Finally, we anticipate beginning RNA and exosome isolation of 12-month samples toward the end of the year.

PROJECTS 3 & 4:

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Early in Year 2, we anticipate receiving final DoD/USAMRMC HRPO approvals, after which we will mail invitations and questionnaires to randomly selected Veterans from the Toxic Embedded Fragment registry (Study Population #1) . We will initiate recruitment and enrollment of Veterans to complete the expanded questionnaire and participate in clinical assessments, to include: Collecting and prepping urine specimens, sending urine specimens for metal and renal marker analyses, and performing PFT and IOS testing at VA recruitment sites (Study Population #2). Available imaging records will be reviewed to determine if fragments have been documented. Additionally, a database will be created and all data will be entered.

4. IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

Nothing to report.

What was the impact on other disciplines?

Nothing to report.

What was the impact on technology transfer?

Nothing to report.

What was the impact on society beyond science and technology?

Nothing to report.

5. CHANGES/PROBLEMS:

<u>John F. Kalinich, Ph.D., Principal Investigator, Project 1</u>
"Health Effects of Embedded Fragments of Military-Relevant Metals"

There were no changes in the objectives and scope of this project. There were modifications made to the pellet implantation surgery schedule to avoid any animal housing issues that might arise during renovation of the Institute's vivarium.

<u>Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2</u>
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report.

<u>PROJECTS 3 & 4:</u>

<u>Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3</u>
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

<u>Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4</u>
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Nothing to report.

Actual or anticipated problems or delays and actions or plans to resolve them.

<u>John F. Kalinich, Ph.D., Principal Investigator, Project 1</u>
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report.

<u>Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2</u>
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report.

<u>PROJECTS 3 & 4:</u>

<u>Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3</u>
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

<u>Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4</u>

"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Although our major tasks for Year 1 were accomplished, we found that the varying schedules of the participating sites' VA Research Committees, as well as the delay in hiring key staff at the Human Resources level, contributed to our delayed submission to the DoD HRPO for final approval. We do not anticipate this being an issue in the future.

Changes that had a significant impact on expenditures

We note a small cost savings in year one of < $20,000 due to a delay in hiring a study coordinator for the Baltimore clinical coordinating site and the identification of newly available freezer space, eliminating the need to purchase a freezer for participant specimens. This savings will be largely offset by the additional cost of purchasing an impulse oscillometer (IO) (type of pulmonary function testing equipment) for the San Antonio site. This purchase was required due to mis-communication with the San Antonio site co- investigator regarding their possession of a functioning IO machine.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.

Significant changes in use or care of <u>human subjects:</u>

<u>Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3</u>
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Nothing to report.

<u>Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4</u>
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Nothing to report.

Significant changes in use or care of <u>vertebrate animals:</u>

<u>John F. Kalinich, Ph.D., Principal Investigator, Project 1</u>
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report.

<u>Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2</u>
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report.

Significant changes in use of biohazards and/or select agents

Nothing to report.

6. PRODUCTS:

- **Publications, conference papers, and presentations**

Journal publications:

<u>John F. Kalinich, Ph.D., Principal Investigator, Project 1</u>
"Health Effects of Embedded Fragments of Military-Relevant Metals"

J.F. Hoffman, A.X. Fan, E.H. Neuendorf, V.B. Vergara, and J.F. Kalinich. Hydrophobic Sand Versus Metabolic Cages: A Comparison of Urine Collection Methods for the Rat (*Rattus norvegicus*). Journal of the American Association of Laboratory Animal Science (submitted). Acknowledgement of federal support – yes.

J.F. Hoffman, V.B. Vergara, S.R. Mog, and J.F. Kalinich. Hydrophobic sand is a non-toxic method of urine collection, appropriate for urinary metal analysis in the rat. Toxics (submitted). Acknowledgement of federal support – yes

<u>Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2</u>
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report

<u>Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3</u>
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Nothing to report.

<u>Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4</u>
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Nothing to report.

Books or other non-periodical, one-time publications.

<u>John F. Kalinich, Ph.D., Principal Investigator, Project 1</u>
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report.

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Nothing to report.

Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Nothing to report.

Other publications, conference papers and presentations.

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report.

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Nothing to report.

Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Nothing to report.

- **Website(s) or other Internet site(s)**

<u>John F. Kalinich, Ph.D., Principal Investigator, Project 1</u>
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report.

<u>Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2</u>
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report

<u>Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3</u>
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Nothing to report.

<u>Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4</u>
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Nothing to report.

- **Technologies or techniques**
Identify technologies or techniques that resulted from the research activities. Describe the technologies or techniques were shared.

<u>John F. Kalinich, Ph.D., Principal Investigator, Project 1</u>
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report.

<u>Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2</u>
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report

<u>Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3</u>
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Nothing to report.

Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Nothing to report.

- **Inventions, patent applications, and/or licenses**

John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Nothing to report.

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Nothing to report

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"

Nothing to report.

Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

Nothing to report.

.

- **Other Products**

PROJECTS 3 & 4:

Questionnaire: "Self-Reported Health Effects in Veterans with Blast and Embedded Metal Fragment Injuries", (Study Population #1)
Project Title: "*Respiratory Health in a Cohort of Embedded Fragment Registry Veterans Exposed to Blasts and Metals"*
Project Leader/PI: Stella Hines, MD, MSPH

Questionnaire: ""Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments", (Study Population #2)
Project Title: *"Biomarker Assessment of Kidney Injury from Metal Exposure in Embedded Fragment Registry Veterans"*
Project Leader/PI: Joanna Gaitens, PhD, MSN/MPH, RN

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

<u>Melissa McDiarmid, M.D., Principal Investigator:</u>
"Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments"

Name: Melissa McDiarmid, M.D.
Project Role: Principal Investigator
Nearest Person Month worked: 2.40
Contribution to Project: Dr. McDiarmid oversaw conduct and progress of all four study projects and participated in quarterly project team call.

Name: Rachel Coates-Knowles, MSM
Project Role: Finance Manager
Nearest Person Month worked: 6.6
Contribution to Project: Maintained and processed all financial transactions and reporting.

Name: Clayton Brown
Project Role: Statistician
Nearest Person Month worked: 2.35
Contribution to Project: Provided input on data collection tools and data design.

Name: Sheila Williams
Project Role: Administrative Assistant
Nearest Person Month worked: 1.20
Contribution to Project: Assist with procurement, travel arrangements, and document preparation.

<u>John F. Kalinich, Ph.D., Principal Investigator, Project 1:</u>
"Health Effects of Embedded Fragments of Military-Relevant Metals"

Name: John Kalinich, PhD
Project Role: Principal Investigator, Project 1
Researcher Identifier: 0000-0003-1591-9389
Nearest person month worked: 2
Contribution to Project: Responsible for overall functioning of this portion of the project.
Funding Support: Federal Government Employee (Department of Defense)

Name: Christine Kasper, PhD RN, FAAN FACS
Project Role: Co-Investigator,
Research Identifier: 0000-0002-7784-2519
Nearest person month worked: 1
Contribution to Project: Responsible for experimental planning
Funding Support: Federal Government Employee (Department of Veterans Affairs)

Name: Anya Fan, MS
Project Role: Research Assistant
Nearest person month worked: 12
Contribution to Project: Responsible for implantation surgeries, urine collection, and animal welfare.

Name: Raisa Marshall, BS
Project Role: Research Assistant
Nearest person month worked: 12
Contribution to Project: Responsible for implantation surgeries and animal welfare. Ms. Marshall has replaced Ms. Neuendorf.

Name: Jessica Hoffman, PhD
Project Role: Co-Investigator
Researcher Identifier: 0000-0003-1858-8394
Nearest person month worked: 5
Contribution to Project: Member of the surgical implantation and euthanasia teams.
Funding Support: Federal Government Employee (Department of Defense)

Name: Co-Investigator, PhD, CDR, USN
Project Role: Local Site Investigator
Nearest person month worked: 1
Contribution to Project: Member of the surgical implantation and euthanasia teams.
Funding Support: U.S. Navy (active duty)

Name: Elizabeth Neuendorf, MSc
Project Role: Research Assistant
Nearest person month worked: 12
Contribution to Project: Responsible for implantation surgeries and animal welfare. Ms. Neuendorf resigned her position on July 24, 2017.

Charlotte A. Peterson, Ph.D., Principal Investigator, Project 2:
"Biomarkers for Assessing Return-to-Duty Potential of Personnel with Embedded Metal-Fragment Wounds"

Name: Charlotte A. Peterson, PhD
Project Role: Principal Investigator, Project 2
Nearest person month worked: 1
Contribution to Project: Responsible for overall functioning of this portion of the project.
Funding Support: University of Kentucky

Name: John J. McCarthy, PhD
Project Role: Co-Investigator
Nearest person month worked: 1
Contribution to Project: Responsible for experimental planning
Funding Support: University of Kentucky

Name: Alexander Alimov
Project Role: Research Scientist II

Nearest person month worked: 2
Contribution to Project: Responsible for exosome isolation and characterization (Western blot analysis) and RNA isolation.

Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Lead Investigator/ Local Site PI, Project 3: ***"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"***

Name: Joanna Gaitens, PhD, MSN/MPH
Project Role: Project Lead Investigator/ Local Site PI
Nearest person month worked: 2.4 person months
Contribution to Project: Responsible for study design and development of protocols; acquired and maintained required approvals; strategized recruitment, enrollment, scheduling, and plans for data and specimen collection; Conducted quarterly project team calls and one in-person meeting

Stella Hines, M.D., MSPH, Project Lead Investigator/ Local Site PI, Project 4: ***"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"***

Name: Stella Hines, MD, MSPH
Project Role: Project Lead Investigator/ Local Site PI
Nearest person month worked: 2.4 person months
Contribution to Project: Responsible for study design and development of protocols; acquired and maintained required approvals; strategized recruitment, enrollment, scheduling, and plans for data and specimen collection; Conducted quarterly project team calls and one in-person meeting

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Nothing to report.

What other organizations were involved as partners?

Participant Enrollment Sites – Clinical Collaboration

Baltimore VAMC (Site 1)
Joanna Gaitens and Stella Hines are the Local Site Principal Investigators for the Baltimore recruitment site. Their contributions to the projects are listed above.

Name: Kate Agnetti, BS
Project Role: Research Coordinator
Nearest person month worked: 6 person months

Contribution to Project: Interacted with HRPO and regulatory bodies in order to obtain and maintain required approvals; assisted in developing recruitment, enrollment, and scheduling strategies, and plans for data and specimen collection; organized and participated in quarterly project team calls and one in-person meeting.

Nashville (Site 2):

Name: Kerri Cavanaugh, MD MHS
Project Role: Local Site Investigator
Nearest person month worked: 1.2 person months
Contribution to Project: Acquired and maintained required approvals; participated in quarterly project team calls and one in-person meeting.

Name: William Lawson, MD
Project Role: Local Site Investigator
Nearest person month worked: 0.6 person months
Contribution to Project: Acquired and maintained required approvals; participated in quarterly project team call; received Impulse Oscillometry training.

Gainesville (Site 3):

Name: Perevumba Sriram, MD
Project Role: Local Site Investigator
Nearest person month worked: 0.6 person months
Contribution to Project: Acquired and maintained required approvals; participated in quarterly project team calls and one in-person meeting.

Name: Nataliya Kirichenko
Project Role: Local Study Coordinator
Nearest person month worked: 6 person months
Contribution to Project: Assisted in acquiring and maintaining required approvals; participated in quarterly project team calls and one in-person meeting; received Impulse Oscillometry training.

Name: Paige Gustad
Project Role: Local Regulatory Assistant
Nearest person month worked: 1.4 person months
Contribution to Project: Interacted with local HRPO and regulatory bodies

Oklahoma City (Site 4):

Name: Lisa Beck, MD
Project Role: Local Site Investigator
Nearest person month worked: 1.8 person months
Contribution to Project: Acquired and maintained required approvals; participated in quarterly project team calls and one in-person meeting.

Name: Vickie Phillips
Project Role: Local Study Coordinator
Nearest person month worked: 6 person months

Contribution to Project: Assisted in acquiring and maintaining required approvals; participated in quarterly project team calls and one in-person meeting; received Impulse Oscillometry training.

San Antonio (Site 5):

Name: Catherine Do, MD
Project Role: Local Site Investigator
Nearest person month worked: 1.2 person months
Contribution to Project: Acquired and maintained required approvals; participated in quarterly project team calls and one in-person meeting.

Name: Antonio Anzueto, MD
Project Role: Local Site Investigator
Nearest person month worked: 1.2 person months annually
Contribution to Project: Acquired and maintained required approval.

Name: Alex Aguilera
Project Role: Local Study Coordinator
Nearest person month worked: 2.0 person months
Contribution to Project: Assisted in acquiring and maintaining required approvals; participated in quarterly project team calls and one in-person meeting; received Impulse Oscillometry training

Name: Myra Mireles
Project Role: Local Study Coordinator
Nearest person month worked: 2.5 person
Contribution to Project: Assisted in acquiring and maintaining required approvals; participated in quarterly project team call; received Impulse Oscillometry training.

8. SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS:

Nothing to report.

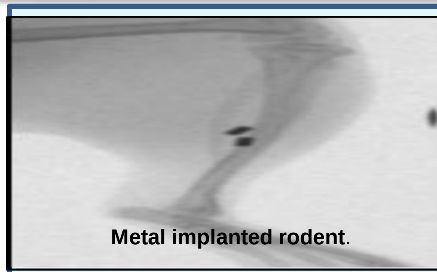
Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments
ERMS/Log Number PR151808
W81XWH-16-2-0058

PI: Melissa McDiarmid, M.D. , M.P.H. **Org:** University of Maryland, Baltimore **Award Amount:** $7,967,578

Study/Product Aim(s)
To provide a scientific evidence base to refine the clinical management of the Veteran or Service member with retained, embedded metal fragments.

Approach

A multidisciplinary approach using animal models and patient data will be used. Simulated metal fragment wounds will be studied using rodents surgically implanted with various metals of toxic concern. In **Project 1**, tissues surrounding the implant will be studied for histopathology, immunochemistry and neoplastic change. **Project 2** will attempt to identify early biomarkers of potential malignant transformation in skeletal muscle, urine and serum from these implanted animals. **Project 3** will assess kidney injury (the presumed target of toxic metal exposure) in Embedded Fragment Registry Veterans and **Project 4**, will assess pulmonary injury in these Veterans both from systemic metal absorption and presumed blast-induced –baro-trauma at the time of injury.



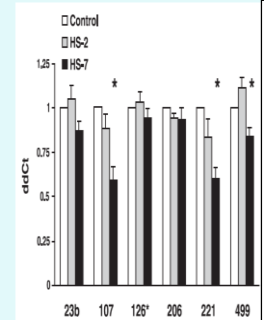


Figure 5. qRT-PCR validation of differentially expressed microRNAs in rat skeletal muscle implanted with metals **(Project 2).** Asterisk denotes significantly ($p < 0.05$) different from control.

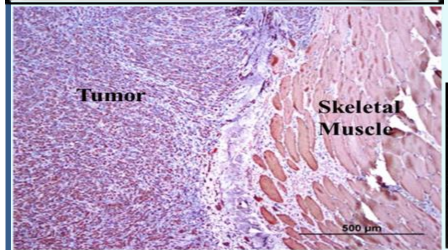
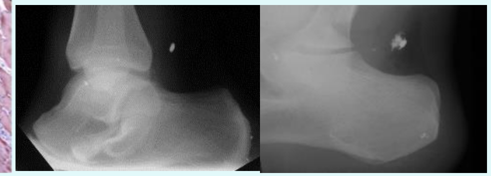


Fig. 2: Desmin-staining of tumor and muscle From metal implant (Project 1)



X-ray of Veteran with embedded metal fragment de-forming (Projects 3 & 4).

Timeline and Cost

Activities CY	2017	2018	2019	2020	2021
PRJ 1: Health Effects of Embedded Fragments of Military-Relevant Metals	100 %				
PRJ 2: Biomarkers for Assessing Return-to-Duty Potential of Personnel	100 %				
PRJ 3: Biomarker Assessment of Kidney Injury from Metal Exposure	100 %				
PRJ 4: Respiratory Health in Cohort of Embedded Fragment Registry Veterans	100 %				
Estimated Budget ($Mil)	**$1.0**	**$1.8**	**$1.9**	**$1.8**	**$1.2**

Updated: October 25, 2017

Goals/Milestones (Example)

Project 1: Animal work approvals secured and rodents implanted.

Project 2: Biomarkers of malignant transformation study protocol optimized with quality control assessments performed.

Meetings with Project PIs – in person and via phone.

Projects 3 & 4: VA Central IRB and local site IRBs obtained.

Exposure and health history questionnaire complete for Cohort 1 (survey only).

Expanded Questionnaire for Cohort 2 (clinical assessment group) complete.

Meetings with Project and Overall PIs and 5 Clinical Assessment site Co-investigators completed.

Comments/Challenges/Issues/Concerns

- Nothing to report.

Budget Expenditure to Date (as of October, 2017)

Projected Expenditure: $1,030,011

Actual Expenditure: $611,335.88

5. APPENDICES

APPENDICES
John F. Kalinich, Ph.D., Principal Investigator, Project 1
"Health Effects of Embedded Fragments of Military-Relevant Metals"

1. Pellet Preparation SOP
2. Pellet Implant Surgery SOP
3. Year 1 Surgery Schedule
4. Figure 1 – Ni tumor photograph
5. Figure 2 – Ni tumor capsule photograph
6. Figure 3 – Cu implantation site photograph
7. Journal of the American Association of Laboratory Animal Science Manuscript
8. Toxics Manuscript

<u>John F. Kalinich, Ph.D., Principal Investigator, Project 1</u>
"Health Effects of Embedded Fragments of Military-Relevant Metals"

<u>PELLET CLEANING SUPPLIES</u>

Sterile pack #1: 2 x 2 gauze (8)
4 x 4 gauze (8)
Glass petri dish with Whatman#1 filter and 4 x 4 gauze (~2 pieces)
Sterile pack #2: Pellet washing baskets (1 per pellet type with 20 pellet max)
Micro-forceps (2)
Sterile pack #3: 50 ml beakers (4)
Sterile pack #4: 13mm borosilicate tissue culture tubes (1 per pellet type; 10-20 pellet max)
Sterile pack #5: Glass vials - 1 per subject
*Pre-weigh sterile vial before adding washed pellets
*If DU pellets mark with rad tape

Chemical hood
Sonicator bath
Timer
24" x 24" absorbent diaper (2)
Nitrile gloves
Safety glasses
100% ethanol, 5-10 ml (Solvent 140, if using DU pellets)
50% nitric acid (approx. 20-25 ml)
70% ethanol (100 ml)
Sterile water (25-30 ml)

Worksheet for pellet vials (pre-weigh sterile, empty vials with caps on)
Waste container for nitric acid
Waste container for mixed rad waste, if using DU pellets

<u>PELLET CLEANING PROCEDURE*</u>

1. Place pellets in 13mm borosilicate tissue culture tube (1 tube per pellet type; 10-20 pellet max).
   Add 1.0 ml 100% ethanol and sonicate for 5.0 minutes (20-23oC)
2. Pour pellets into dipping basket (≤ 20 pellets per basket). Rinse pellets with 70% ethanol.
3. Place pellets in 50% nitric acid for 3 minutes (agitate occasionally).
   -store used nitric acid in waster container for proper disposal at a later time
4. Rinse pellets with sterile water.
5. Rinse pellets with 70% ethanol.
6. Allow pellets to air dry.
7. Count pellets into pre-weighed sterile vials.
8. Weigh vials containing counted pellets.
9. Add 1-2 ml 70% ethanol to submerge/coat pellets.
   (At time of surgery, rinse pellets with 0.9% saline while on Whatman #1 filter paper, before implanting.)

*When using DU pellets:
-wash all non-rad pellets first (DU pellets last) to avoid rad contamination
-be sure to label all relevant items with rad tape
-ALL wash solutions must go in rad labeled waste container (pH and rinse down warm drain)
-all used PPE, etc. must go in rad waste

Pellet Implantation Surgery SOP

Day prior to surgery
Collect and assemble a new clean cage system for each surgery subject from the vivarium
Cage system includes: appropriate box with bedding, Nyla bone and rodent toy cage card holder
appropriate wire rack with rodent chow and water bottle, and filter top
Program microchips and double check for correct code
Count out proper type and number of pellets
Prepare correct dilution and volume of buprenorphine (drug safe code: 2 & 4 simultaneously, then 3)

Day of surgery
In the lab (part 1):
Sterilize and prepare all pellets needed
Draw saline for pellet rinse (1-2 10 ml syringes)
Turn on all 3 heating pads (prep, surgery, recovery)
Fill vaporizer with isoflurane and open oxygen tank valve (check psi)
Prep Vetbond, buprenorphine, 1 ml syringes and #10 scalpel blades

In the vivarium, weigh each subject and place them into a clean cage with appropriate cage card

In the lab (part 2):
Calculate buprenorphine dose needed for each subject
With Fluovac absorber on, set oxygen flow to1.0 L/min and isoflurane to 3-5 % MAC

Place subject into induction chamber until sedated
When subject is sedated, transfer to nose cone in surgery prep area (reduce isoflurane to 2-4% MAC)
- clamp unused nose cone line
- remove induction chamber from isoflurane flow pathway → directly connect input and output lines
- adjust MAC

Clean appropriate ear with 70% isopropyl alcohol (IPA) and ear punch
Prep microchip site with IPA and implant microchip along mid-dorsal line, seal with Vetbond
Prep analgesia site with 70% isopropyl alcohol, then administer buprenorphine subcutaneously with a 25 gauge, 1” syringe

Closely clip implantation sites, remove all hair, clean clipped area with isopropyl alcohol and betadine
Prep/open surgical pack (remove pellet loading gear and open drape)
Carefully transfer subject to nose cone in prepared surgical area → do not contaminate incision sites
Move anesthesia line clamp to opposite nose cone

Adjust isoflurane MAC (2-4%)

Make incision with #10 blade over gastrocnemius
Inject pellets into muscle tissue using 14 or 16 gauge needle and plunger (one at a time)
Repeat incision and pellet injection steps on second hind limb

Seal both incisions with Vetbond and move subject to recovery chamber
Observe until ambulatory and return to home cage.
Observe for 2-4 hours, record body temperature and return to vivarium.

After surgery clean all work areas and equipment
Weigh Fluovac canister and record on adsorber canister (dispose of canister at 1400 grams)
Clean clippers in Blade Wash, wipe down with isopropyl alcohol, then spray on Clippercide

MAY 2017

SUN	MON	TUES	WED	THURS	FRI	SAT
	1 LabSand Exp Round 2 2 h	2	3 LabSand Exp 4 h	4	5 LabSand Exp 6 h	6
7	8	9 LabSand Exp 6 h	10	11 LabSand Exp 6 h	12	13
14	15	16 Deliver 3M Rats (16) – (Ta/W)	17 Practice Rats – Implant Surgery (4)	18 Practice Rats – Implant Surgery (4)	19	20
21	22	23 Deliver 3M Rats (16) – (Ni/Co)	24 Pair house Practice Rats (4)	25 Pair house Practice Rats (4)	26	27
28	29 **MEMORIAL DAY**	30 Deliver 3M Rats (16) – (Fe/Cu) LabSand – 3M Ta (8)	31			

JUNE 2017

SUN	MON	TUES	WED	THURS	FRI	SAT
				1 **LabSand – 3M W (8)**	2	3
4	5 Implant 3M Rats (8) – Ta Order 12M Rats (6 weeks old)	6 Deliver 3M Rats (16) – (Al/Pb) LabSand – 3M Ni (8)	7 Implant 3M Rats (8) - W	8 LabSand – 3M Co (8)	9	10
11	12 Implant 3M Rats (8) – Ni Pair house 3M Ta	13 Euthanasia – Practice Rats (4) Deliver 3M Rats (8) – (DU) LabSand – 3M Fe (8)	14 Implant 3M Rats (8) – Co Pair house 3M W	15 Euthanasia – Practice Rats (4) LabSand – 3M Cu (8)	16	17
18	19 Implant 3M Rats (8) – Fe Pair house 3M Ni	20 LabSand – 3M Al (8)	21 Implant 3M Rats (8) – Cu Pair house 3M Co	22 LabSand – 3M Pb (8)	23	24
25	26 Implant 3M Rats (8) – Al Pair house 3M Fe	27 Deliver 12M Rats (16) – (Ta/W)	28 Implant 3M Rats (8)– Pb Pair house 3M Cu	29 LabSand – 3M DU (8)	30	

JULY 2017

SUN	MON	TUES	WED	THURS	FRI	SAT
						1
2	3 Pair house 3M Al	4 **INDEPENDENCE DAY**	5 Implant 3M (8) – DU Pair house 3M Pb Deliver 12M Rats (16) – (Ni/Co)	6	7	8
9	10	11 Deliver 12M Rats (16) – (Fe/Cu) LabSand – 12M Ta (8)	12 Pair house 3M DU	13 LabSand – 12M W (8)	14	15
16	17 Implant 12M (8) – Ta	18 Deliver 12M Rats (16) – (Al/Pb) LabSand – 12M Ni (8)	19 Implant 12M (8) - W	20 LabSand – 12M Co (8)	21	22
23/30	24 Implant 12M (8) – Ni Pair house 12M Ta 31 Implant 12M (8) – Fe Pair house 12M Ni	25 Deliver 12M Rats (8) – (DU) LabSand – 12M Fe (8)	26 Implant 12M (8) – Co Pair house 12M W	27 LabSand – 12M Cu (8)	28	29

AUGUST 2017

SUN	MON	TUES	WED	THURS	FRI	SAT
		1 LabSand – 12M Al (8)	2 Implant 12M (8) – Cu Pair house 12M Co	3 LabSand – 12M Pb (8)	4	5
6	7 Implant 12M (8) – Al Pair house 12M Fe	8 LabSand – 12M DU (8)	9 Implant 12M (8) – Pb Pair house 12M Cu	10	11	12
13	14 Implant 12M (8) – DU Pair house 12M Al	15	16 Pair house 12M Pb	17	18	19
20	21 Pair house 12M DU Order 1M Rats (6 weeks old)	22	23	24	25	26
27	28	29 LabSand – 3M Ta (4)	30 LabSand – 3M Ta (4)	31 LabSand – 3M W (4)		

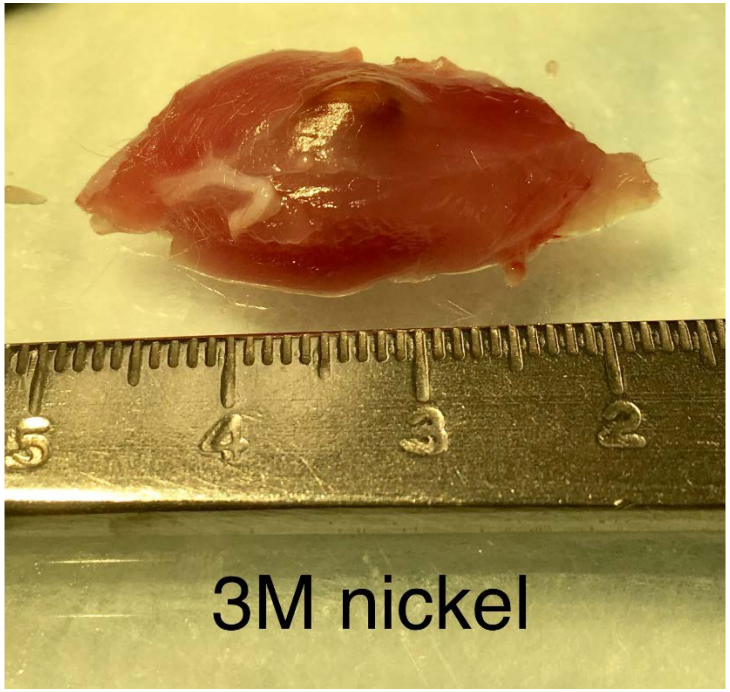


Figure 1: Nickel-induced tumor. Photograph shows tumor in the gastrocnemius muscle of a male Sprague Dawley rat implanted with a nickel pellet (1mm x 2 mm) for 3 months.

Figure 2: Nickel-induced tumor. Photograph shows tumor surrounding an implanted nickel pellet (1 mm x 2 mm). Pellet had been surgically implanted in the gastrocnemius muscle of a male Sprague Dawley rat 3 months earlier.

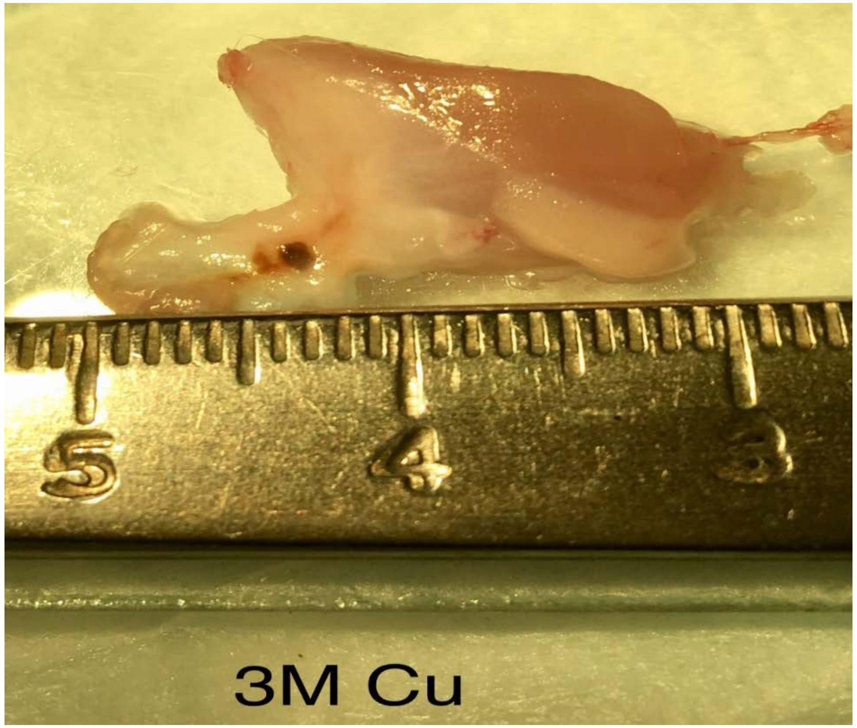


Figure 3: Remnants of implanted copper pellet (1 mm x 2 mm). Pellet had been surgically implanted in the gastrocnemius muscle of a male Sprague Dawley rat 3 months earlier.

Hydrophobic Sand Versus Metabolic Cages: A Comparison of Urine Collection Methods for the Rat (*Rattus norvegicus*)

Jessica F. Hoffman[1,*], Anya X. Fan[1], Elizabeth H. Neuendorf[1], Vernieda B. Vergara[1], John F. Kalinich[1]

[1] Internal Contamination and Metal Toxicity Program, Armed Forces Radiobiology Research Institute, Uniformed Services University, Bethesda, MD

**Corresponding author. Email: jessica.hoffman@usuhs.edu*

Comparison of Urine Collection Methods in the Rat

Abstract

A commonly used method for urine collection from the rat requires the use of a metabolic cage, subjecting animals to extended periods of isolation in an unfamiliar cage with a wire mesh floor. Recently, a new method involving hydrophobic sand, a material more similar to bedding, has become available, but has not been extensively tested for collection efficiency or stress compared to the metabolic cage. Using a within-subjects crossover design, we examined differences in stress response, urinary markers, and urine volume for 2, 4, and 6 hour collection sessions in hydrophobic sand and metabolic cages in male Sprague Dawley rats. We found no significant differences between hydrophobic sand and metabolic cages in stress response markers of weight loss, fecal pellet output, or corticosterone, and observed behavior indicates sand may be less stressful than the metabolic cage. All clinically relevant urinary markers examined were normal with no differences between collection methods. Total urine volume collected was greater from the metabolic cage than sand in 3 of the 5 sessions, but the shortest session (2 hours) had no significant difference in volume between methods and resulted in more than half (61.93%) of the total volume collected. Our results suggest hydrophobic sand is a refinement of rat urine collection methods, capable of reducing isolation time, risk of injury, and stress without compromising urine sample integrity.

Abbreviations and Acronyms:

LS	LabSand (a specific brand of hydrophobic sand)
MC	Metabolic Cage

Introduction

Collection of urine samples from rodents in a volume sufficient for standard urinary testing protocols usually involves single housing the rodents for an extended period of time, commonly 16 to 24 hours, in metabolic cages. While not considered overly stressful for the animal[6,5], a period of habituation to the metabolic cage is recommended[8] and the collection procedure requires removal of the animal from its normal home cage environment. Animal ethics review guidelines recommend that animals not be housed in metabolic cages without express permission of the Animal Ethics Committee of the institution, and all efforts to enrich the cage and provide rats with visual, auditory, and olfactory contact with other rats as far as possible[1]. Recently, a product developed to permit non-stressful urine collection from cats has been proposed as a potentially useful way to collect urine from rodents as well. Hydrophobic sand is a biodegradable material with a non-toxic urine-repelling coating, currently available as “LabSand” to the scientific community, or “Kit4Cat” commercially. Hydrophobic sand replaces the bedding in a normal cage during the urine collection period. After collection is complete, the rats can be returned to their normal home cage environment and the used Kit4Cat/LabSand disposed as laboratory waste.

Although there are no reports in the peer-reviewed literature using this material for rodent urine collection, a poster presentation from the Laboratory Animal Science and Safety Assessment Group at GSK[12] compared metabolic cages and the Kit4Cat hydrophobic sand, assessing for urine collection volume and urinalysis integrity in mice. They found that 3 hour collections from the sand yielded their necessary volume (0.2 ml) in 85% of mice, and there were no significant differences in 10 urinalysis markers between 3 hour collections from sand and 16 hour collections from metabolic cages. However, they did not measure any stress markers in the mice, nor did they directly compare the same amounts of time in sand verses metabolic cages. The literature search also found an abstract from a JAALAS conference[10] comparing volume collections from hydrophobic sand and metabolic cages at various time points in both mice and rats. They report that for mice with collection times of 3, 6, and 24 hours in either sand or metabolic cages, urine volume was significantly less in sand than metabolic cage only at the 24 hour collection time. For rats with collection times of 2, 4, and 6 hours, urine volume was significantly less in sand than metabolic cages for all collection times, though the abstract did not describe actual volumes collected. Further, their study lacked any comparisons of stress or urinalysis assays between collection methods.

Corticosterone is a main glucocorticoid hormone produced in the adrenal gland in rodents and serves as a primary stress response; the human equivalent is cortisol[13]. Urinary corticosterone levels are an accepted measure of stress response in the rodent[2,7]. In addition, the number of fecal pellets expressed during urine collection is also considered a marker of stress[3,4,11]. The goal of the current study was to determine if the use of hydrophobic sand can provide a useful urine sample from rats without the stress associated with metabolic cage housing. Our hypothesis was that measures of stress during urine collection via hydrophobic sand would be either not

significantly different than, or significantly less than, during urine collection via metabolic cage. Additionally, we expected to see no evidence of contamination from the sand that would affect clinically relevant urine marker measurements and properties in future studies.

Materials and Methods

Test subjects and housing conditions. Experiments in this study were conducted at the Armed Forces Radiobiology Research Institute (AFRRI). Male Sprague Dawley rats (Rattus norvegicus, n=8) approximately 30 days old, 75-100 g, were purchased from Envigo (Barrier 208A, Frederick, MD). Rats were allowed to acclimate in the vivarium for a minimum of 2 weeks prior to the start of experiments. The room was maintained at standard temperature and humidity (21 ± 2 ^{o}C, 30% to 70%) with alternating 12:12 light:dark cycle (lights on, 0600 h) and access to Teklad Global Rodent Diet 8604 (Envigo) and water *ad libitum*. Cages were changed 2-3 times weekly. The rats were pair-housed in plastic microisolator cages (23.8 x 45.4 cm) on Teklad Sani-Chips bedding (Envigo) and individually in Nalgene metabolic cages (Thermo Fisher, Pittsburgh, PA) or smaller (mouse) plastic microisolator cages (described below) on hydrophobic sand during urine collection. All procedures involving animals were (a) conducted with maximum possible well-being of the rats, (b) approved by the AFRRI Institutional Animal Care and Use Committee prior to the start of the study (Protocol #: 2016-05-006), and (c) performed in compliance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC-I).

Urine collection apparatus. For both collection methods, animals were single-housed for the duration of the session, and immediately returned to pair housing in their home cages at the end of the session. Rats had free access to water replacement pouches (HydroGel®, Clear H_2O, Westbrook, ME) instead of water bottles to avoid dilution of urine droplets in the hydrophobic sand. All cages were cleaned thoroughly with Contrex detergent and water between sessions.

<u>Metabolic cage:</u> Nalgene metabolic cages were used. The cage consists of a circular upper portion which houses the rat, a wire grid floor (diameter 21.5 cm, surface area appx 363 cm^2, with openings of 1 cm by 3.1 cm) the rat must stand on, and a lower collection chamber with a specialized funnel that separates fecal pellets and urine that fall through the grid floor to collect into two separate Nalgene tubes 4 cm in diameter.

<u>Hydrophobic sand:</u> 300 g (single pack) of LabSand (Coastline Global, Inc., Palo Alto, CA) was spread around the bottom of a mouse plastic microisolator cage (15.2 x 25.4 cm, surface area 386 cm^2) with a filtered lid.

Urine pools on top of the sand, and was collected with a pipette at specific time intervals (see schedule of collection).

Group assignment and schedule of collection. The experimental design and urine sample collection schedule is illustrated in Figure 1. In a within-subjects crossover design, rats were randomly assigned to either Group A (metabolic cage followed by LabSand, n=4) or Group B (LabSand followed by metabolic cage, n=4). Since a habituation period is highly recommended when using metabolic cages, both methods followed the procedure utilized for our previous studies[9] involving urine collection. Groups A and B were run simultaneously in the same testing room. There were a total of 5 collection sessions for each method: 2 hours, 4 hours, and three 6 hour sessions. Each session was separated by a rest period of at least 48 hours over a period of 2 weeks, at which point the session schedule was repeated, but with animals switching collection method. Sessions began at 0800 h each day, and testing room lighting and temperature was maintained at the same level as that in the regular husbandry housing room.

Rats were weighed prior to and after each session, and each rat's total fecal pellets were counted for each session. In the metabolic cages, the urine collection tube is graduated in 2 ml increments, but urine volume can only be determined at the end of the collection period. For the lab sand, urine can be collected at any time; output volume was determined at every 30 min during the session, and pooled at the end. Refractometer and test strip analyses were completed immediately on all pooled urine samples before storage at -80^{o}C until further analysis. All frozen samples were analyzed in a single session.

Urinalysis. Multiple methods were used to assess normal urinary markers of general health and stress. A Digital Refractometer 300027 (Kernco, El Paso, TX) was used to determine urine specific gravity (USG; detection range 1.000 – 1.050); and refractive index (nD; detection range 1.3330 to 1.3900). Clinically-relevant urine markers were assessed by URS-10T test strips (HealthyWiser, Eastleigh, Hampshire, United Kingdom). Each test strip consists of colorimetric reaction spots for 10 individual markers: leukocytes (range: negative to 500 cacells/µl), nitrite (negative or positive), urobilinogen (range: 3.2 – 125 µmol/l), protein (range: negative to >20.2 g/l), pH (range 5.0 – 8.5), blood (negative, trace of non-hemolyzed, or hemolyzed 10-220 cacells/µl), specific gravity (range 1.000 – 1.030), ketone (range: negative – 16 mmol/l), bilirubin (range: negative – 100 µmol/l), and glucose (range: negative – 110 mmol/l). Each square is wet with a droplet of urine and the marker value is determined against a standard association chart after the required amount of reaction time (30 -120 s). The urine sticks were assessed by eye by two technicians.

Creatinine. Urine creatinine levels were determined with a colorimetric creatinine assay kit (Cat# CR01, Oxford Biomedical Research, Inc., Oxford, MI) read on a spectrophotometer (SpectraMax 190, SoftMax Pro 2.0 software, Molecular Devices, Sunnyvale, CA). Briefly, urinary creatinine produces an orange color when it reacts with picric acid under alkaline conditions. This reaction also occurs with other components in biological fluids, but the specific color produced by creatinine degrades rapidly under acidic conditions. Urine samples are diluted, placed on a 96-well plate, picric acid added, and the color reaction read at 490 nm. Acid reagent is then added and the reaction read again at 490 nm. The difference in absorbance reading is calculated, samples values determined against a creatinine standard curve (0 – 10.0 mg/dl), and corrected for dilution.

Corticosterone. Urine corticosterone levels were determined with a colorimetric corticosterone ELISA (enzyme-linked immunosorbent assay) kit (Cat# ab108821, Abcam, Cambridge, MA; minimum detectable dose 0.28 ng/ml). Briefly, diluted urine samples are added to a 96-well plate precoated with a corticosterone specific antibody. Biotinylated corticosterone is added to each well, then washed with wash buffer. Streptavidin-peroxidase conjugate is added to each well, and unbound conjugates are washed away with wash buffer. A chromogen substrate is added to each well to produce a blue color, which changes to yellow after an acidic stop solution. The plate is then read at 450 nm on a spectrophotometer (Spectramax 190), sample values determined against a corticosterone standard curve (0 – 100 ng/ml), and corrected for dilution.

Statistical analysis. Animal growth over time was determined by a line of best fit for each group's growth, and subjected to a comparison of fits (Group A versus Group B). Decrease in urine corticosterone over time was determined by a line of best fit for each group's growth, and subjected to a comparison of fits (metabolic cage versus LabSand). Unless specifically noted, all other data was analyzed as a within-subjects two-tailed t-test comparison between collection methods for each session time. All analyses used GraphPad Prism Software (version 7.01, La Jolla, CA). P values less than 0.05 were considered significant.

Results

Urinalysis. There were no significant differences in refractive index or specific gravity between the metabolic cage and LabSand collection method during any session (Table 1). All common urinalysis clinical markers assessed by URS-10T test strips were within normal range for all animals. There was no variability in nitrate (all tests negative), urobilinogen (all tests 3.2 µmol/l), or blood (all negative). Bilirubin was negative for all tests except 8 of 80, all of which measured at 17 µmol/l, with no group pattern difference. Glucose was negative

for all tests except 1 of 80, which measured at 15 mmol/l. There were no significant differences in leukocytes, protein, pH, ketones, or creatinine between metabolic cage and LabSand collection methods during any session (Table 1).

Urine volume collection. Total urine volume collected from metabolic cages compared with LabSand was not significantly different by the end of the 2 hour session ($t_7 = 1.002$, $P = 0.35$) or the second 6 hour session ($t_7 = 1.07$, $P = 0.32$), but did result in a significantly higher volume yield by the end of the 4 hour ($t_7 = 4.43$, $P < 0.01$) and first and third 6 hour sessions ($t_7 = 4.47$, $P < 0.01$; $t_7 = 4.47$, $P < 0.01$, respectively) (Figure 2A).

Due to the style of the metabolic cage collection tube, it was not possible to assess urine output throughout each session. However, urine was able to be collected frequently from LabSand (every 30 minutes), and is graphed as cumulative urine volume over time in Figure 2B to assess the pattern of urine output over time. The rate of urine output slows over time and is not a linear increase. More than half of the total volume was collected within the first two hours. The average cumulative volume collected at 2 hours (all 5 sessions) was 1.15 ml (SD = 0.62). The 2 hour collective volume represents 69.5% (SD = 29.75%) of the total volume (all 5 sessions). Excluding the 2 hour session, the 2 hour collective volume for the other four sessions represents 61.93% (SD = 28.54%) of the total volume.

Stress assessment. There was no significant difference in initial weight between the two groups (Group A: mean = 236.4g, SD = 2.47, Group B: mean = 240.2g, SD = 1.95; $t_6 = 2.43$, $P = 0.05$). Weight gain in animals over the course of the entire experiment was normal. Growth over time was not significantly different between the groups (Group A: Y-int = 235.3, slope = 3.96, $R^2 = 0.971$; Group B: Y-int = 239.7, slope = 4.15, $R^2 = 0.974$; comparison of fits: $F_{(1,76)} = 1.491$, $P = 0.24$). Weight lost within each session was not significantly different between metabolic cage and LabSand collection methods for any session (2hr: $t_7 = 0.86$, $P = 0.42$; 4hr: $t_7 = 1.65$, $P = 0.14$; 6hr-1: $t_7 = 0.29$, $P = 0.78$; 6hr-2: $t_7 = 0.97$, $P = 0.36$; 6hr-3: $t_7 = 0.35$, $P = 0.73$) (Figure 3A).

There were no significant differences in total fecal pellet counts between metabolic cage and LabSand collection methods in the 4 hour session ($t_7 = 1.02$, $P = 0.34$) or any of the 6 hour sessions ($t_7 = 1.02$, $P = 0.33$; $t_7 = 1.08$, $P = 0.32$; $t_7 = 0.47$, $P = 0.66$, respectively). In the 2 hour session, the LabSand pellet count (mean = 4.5, SD = 3.5) was significantly higher than the metabolic cage pellet count (mean = 1.5, SD = 2) (Figure 3B, $t_7 = 3.31$, $P = 0.01$). However, this difference is due to a single rat with a much higher pellet count than the rest of the group (Dixon's test for a single outlier, $P < 0.05$).

There were no significant differences in urine corticosterone concentrations between metabolic cage and LabSand collection methods in any of the sessions (2 hr: $t_7 = 0.70$, $P = 0.51$; 4hr: $t_7 = 0.20$, $P = 0.85$; 6hr-1: $t_7 =$

1.07, P = 0.32; 6hr-2: t_7 = 0.71, P = 0.50); 6hr-3: t_7 = 0.71, P = 0.50) (Figure 3C). Additionally, corticosterone levels decreased for all subjects over subsequent sessions, but with no significant difference in the rate between metabolic cage and LabSand collection methods (metabolic cage: Y-int = 20.77, slope = -1.421, R^2 = 0.285; LabSand: Y-int = 23.78, slope = -1.575, R^2 = 0.369; comparison of fits: $F_{(1,76)}$ = 0.087, P = 0.77).

During each session, animal behavior was observed but not quantified. Rats in metabolic cages did not exhibit overt signs of stress, but appeared less ambulatory and with greater difficulty walking due to the wire grid floor (Figure 3D). Rats appeared more relaxed in the LabSand cages, exhibiting normal exploratory and grooming behavior similar to that seen in home cages with normal bedding (Figure 3E). Often, in the metabolic cage rats would nap or rest with their heads tucked under their chests in an attempt to get their paws off the grid (Figure 3F); in the LabSand cage, rats rested normally, curled in a C shape. (Figure 3G). Additionally, all rats consumed some of the available hydrocup, and in the later sessions treated it as an enrichment toy, often flipping it over and standing on it. All rats displayed normal behaviors upon return to their home cage.

Discussion

The metabolic cage is currently one of the few approved, and most commonly used, methods of collecting urine from the laboratory rodent. While effective, its use must be justified due to the potential for the long isolation, unfamiliar shape, and wire bottom inducing stress or injury to the animal. A new alternative urine collection method, hydrophobic sand, has recently come to the market, but little research has been published on its use, effectiveness, or stress in rodents. To our knowledge, this study represents the first peer-reviewed publication comparing use of hydrophobic sand to the metabolic cage in the rat as a potential refinement of urine collection methods.

Our main goal was to determine whether hydrophobic sand would be a successful alternative method of urine collection in the rat instead of the traditional metabolic cage. Our condition for hydrophobic sand qualifying as “successful” was a minimum of not being significantly different than metabolic cages in 1) normal urinary markers/properties, 2) urine volume collection, and 3) measures of stress for the rat. Due to the many small pieces in the metabolic cages that must be assembled, disassembled, and cleaned between each use, the ability to use an alternate method with a faster set-up, easier clean-up, and no additional stress to the rat for the same quantity and quality of urine collection would be very important. If hydrophobic sand proved to also be less stressful and/or more efficient for urine collection, that would provide even more reason to use the alternative method to metabolic cages. To accomplish this we employed a within-subjects crossover design so each rat would serve as its own

control comparing the two collection methods, increasing our statistical power while minimizing the number of animals needed for the study.

The most important of the criteria for using hydrophobic sand in future studies instead of metabolic cages is ensuring that the sand creates no greater level of stress to the animals than the metabolic cages. Neither Smith et al. (mice only) or Pinkus et al. (mice and rats) analyzed stress-specific differences in their sand versus metabolic cage collection experiments. Hydrophobic sand and metabolic cages present two structurally different environments for the rat. The mouse cages we used for the sand had a floor space of 386 cm^2, in a familiar rectangular shape, and the texture of the sand similar to the texture of regular bedding, and poses no risk of injury to small feet. In the metabolic cage, rats had less floor space (363 cm^2), an unfamiliar circular shape with no corners to huddle in, and a wide wire mesh floor they have to learn to navigate or risk getting a foot caught in the grid. Hydrocups were included in both collection methods in every session to serve as a source of hydration instead of a water bottle that could potentially dilute urine. The cups also served as a form of enrichment to counteract the isolation required for both methods. From the exploratory and resting behavior we observed, in the opinion of experienced animal researchers, the rats were more comfortable and relaxed in the sand environment than the metabolic cage. In addition to observed behaviors, we quantified 3 different common measures of stress response: weight loss, fecal pellet counts, and corticosterone in urine. Both crossover groups had the same initial weights and the same growth weight over the course of the entire experiment, so all animals were normal. Neither method induced rapid weight loss as there were no significant differences in any session between sand and metabolic cage for weight lost during the collection period. Although the 2 hour session had significantly higher fecal pellet counts in the sand group, indicating greater stress, this difference was due to a single outlier and there were no differences in fecal pellet count between sand and metabolic cage for any other session. Corticosterone, a hormone produced by the adrenal gland, is recognized as positively correlating to stress level in the rat. There were no significant differences in urine corticosterone concentrations between sand and metabolic cage for any session. We also note that corticosterone decreased over time (across repeated exposure and longer session times) for both groups, with no significant difference in the slope, indicating rats habituated equally to each urine collection method. Our results suggest using hydrophobic sand as a urine collection method induces no more stress than metabolic cages, and based on the behavior, potentially provide a less stressful environment that is too subtle to elicit a change in corticosterone response.

The next important comparison between sand and metabolic cage methods of urine collection is the quality of the urine, ensuring there are no differences in clinically relevant urine markers or properties that would create a confounding variable in future studies. The study by Pinkus et al. did not examine any urine marker analysis, and the study by Smith et al. reported no significant differences in 10 basic urinary markers. We compared urine

properties (refractive analysis and specific gravity) as well as several urinary markers (creatinine, leukocytes, protein, pH, ketones, nitrate, urobilinogen, blood, bilirubin, and glucose) and found no significant differences between using sand or metabolic cages for urine collection for any session. Although the urine tests are not considered as accurate as more advanced diagnostic techniques, our results suggest the use of hydrophobic sand does not introduce any contaminants or alter urine properties in any way that would be relevant to future studies using urine for analysis.

The final determination of success of hydrophobic sand as an alternative method for urine collection is its efficiency compared to the metabolic cage procedure, i.e., the ability to collect a useable volume of urine in the same amount of time as metabolic cages. We examined this in two ways: comparing total volume collected across methods for each session, and calculating cumulative urine collected over time within each session. When comparing across methods within a session, we found significantly higher total urine volume from metabolic cages than sand in 3 of the 5 sessions (4 hours, and the first and third 6 hour sessions). There was no difference in urine volume between methods for the other two sessions. The structure of the metabolic cage and the urine collection tube does not allow for urine volumes to be determined at any time other than the end, therefore no cumulative totals over time are reported. With hydrophobic sand, however, urine is easily collected at any time because it pools on the surface and can be removed with a pipette. We collected urine from sand subjects every half hour for the duration of each session and reported this as cumulative volume collected for each session. While volume is greater in the longer sessions than the shorter ones, as would be expected, we found that greater than half (almost 62%) of the total urine is collected within the first two hours of the session, providing us with an average of 1.15 ml of urine per subject. Depending on the volume needed for subsequent analyses, a single two hour session, or several two hour sessions spaced over several days, should be sufficient to maximize the amount of urine collected from each rat while minimizing their time spent in isolation away from the home cage, especially since there was no difference in total urine volume collected between sand and metabolic cages for the two hour session. Similarly, Pinkus et al. reported that mice had lower total urine volumes in the sand than the metabolic cages only during a 24 hour session as compared to 3 or 6 hour sessions, while rats had lower total urine volumes in sand at all sessions examined (2, 4, and 6 hours). However, they only collected volume at the end of each session, not every half hour, and they noted that they observed rats drinking the urine droplets. We also observed rats ingesting urine in between half hour collection times, while rats in the metabolic cages have no access to excreted urine. Therefore, if urine was collected from the sand as it was deposited rather than at set collection times, we would expect total urine volume collected during a session would be higher than what we report here.

Together our data suggest hydrophobic sand is a viable alternate method of urine collection in the laboratory rat that does not alter important urine properties or induce any more stress than the currently accepted

method using metabolic cages. For studies that require greater volumes of urine, sand can be repeated multiple days in shorter sessions rather than a continuous single session in a metabolic cage, reducing extended periods of isolation. Investigations with urinary metabolites that exhibit diurnal variation would also benefit from using the hydrophobic sand method, as more precise timing of sample collections could be easily performed. Additionally, hydrophobic sand is easily discarded as laboratory waste and does not require the extensive disassembly and cleaning that metabolic cages require, and may prove more cost effective than purchasing a large number of metabolic cages.

Acknowledgements

The project described was supported by the grant Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments (W81XWH-16-2-0058) from the Congressionally Directed Medical Research Programs (CDMRP) Peer Reviewed Medical Research Program.

All procedures involving animals were (a) conducted with maximum possible well-being of the rats, (b) approved by the AFRRI Institutional Animal Care and Use Committee prior to the start of the study under protocol 2016-05-006, and (c) performed in compliance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

The use of the LabSand brand of hydrophobic sand in this work does not represent an endorsement of the product or the company by the U.S. Government.

The views expressed in the paper are those of the authors and do not reflect the official policy or position of the Armed Forces Radiobiology Research Institute, the Uniformed Services University, the Department of Defense, or the United States Government.

References

1. **Animal Research Review Panel.** 2007. [Internet]. Guideline 20: Guidelines for the housing of rats in scientific institutions. [Cited 26 May 2017]. Available at http://www.animalethics.org.au/__data/assets/pdf_file/0014/222512/housing-rats-scientific-institutions.pdf
2. **Bamberg E, Palme R, Meingassner JG.** 2001. Excretion of corticoid metabolites in urine and faeces of rats. Laboratory Animals **35**: 307-314.
3. **Barone FC, Barton ME, White RF, Legos JJ, Kikawa H, Shimamura M, Kurtani K, Kinoshita M.** 2008. Inhibition of phosphodiesterase type 4 decreases stress-induced defecation in rats and mice. Pharmacology **81**: 11-17.

4. **Cavigelli SA, Guhad FA, Ceballos RM, Whetzel CA, Nevalainen T, Lang CM, Klein LC.** 2006. Fecal corticoid metabolites in aged male and female rats after husbandry-related disturbances in the colony room. Journal of the American Association for Laboratory Animal Science **45**: 17-21.
5. **Eriksson E, Royo F, Lyberg K, Carlsson H-E, Hau J.** 2004. Effect of metabolic cage housing on immunoglobin A and corticosterone excretion in faeces and urine of young rats. Experimental Physiology **89**: 427-433.
6. **Gil MC, Aguirre JA, Lemoine AP, Segura ET, Barontini M, Armando I.** 1999. Influence of age on stress responses to metabolic cage housing in rats. Cellular and Molecular Neurobiology **19**: 625-633.
7. **Jorgensen A, Maigaard, Wortwein G, Hageman I, Henriksen T, Weimann A, Moller P, Loft S, Hau J, Poulsen HE, Jorgensen MB.** 2013. Chronic restraint stress in rats causes sustained increase in urinary corticosterone excretion without affecting cerebral or systemic oxidatively generated DNA/RNA damage. Progress in Neuro-Psychopharmacology and Biological Psychiatry **40**: 30-37.
8. **Kurien BT, Everds NE, Scofield RH.** 2004. Experimental animal urine collection; a review. Laboratory Animals **38**: 333-361.
9. **Mak TD, Tyburski JB, Krausz KW, Kalinich JF, Gonzalez FJ, Fornace Jr. AJ.** 2015. Exposure to ionizing radiation reveals global dose- and time-dependent changes in the urinary metabolome of rat. Metabolomics **11**: 1082-1094.
10. **Pinkus CA, Pereira A, Nguyen A, Debrue MC, Menendez LG.** 2016. Use of hydrophobic sand as a refinement for urine collection in mice and rats. JAALAS, Abstracts of Scientific Presentations **50**(5): 606-710.
11. **Smith AL, Leung J, Kun S, Zhang R, Karagiannides I, Raz S, Lee U, Golovatscka V, Pothoulakis C, Bradesi S, Mayer EA, Rodriguez LV.** 2011. The effects of acute and chronic psychological stress on bladder function in a rodent model. Urology **78**: 967.e1-967.e7.
12. **Smith JC, Lancaster H, Ridley D, McClure F, Whelan G**. [Internet]. 2015. 3Rs Refinement – Use of hydrophobic sand in collection of analytical urine samples [Cited 26 May 2017]. Available at http://labsand.com/wp-content/uploads/2015/05/GSK-poster.pdf
13. **Tulane University, e.hormone.** [Internet]. 2014. The Hormones: Corticoids. [Cited 26 May 2017]. Available at http://e.hormone.tulane.edu/learning/corticoids.html

Figure Legends

Figure 1. Experimental design. Rats were randomly assigned to either Group A (metabolic cage followed by LabSand, n=4) or Group B (LabSand followed by metabolic cage, n=4) and run simultaneously in a within-subjects crossover design. Five collection sessions with increasing length of time (2, 4, 6, 6, and 6 hours, respectively) were run over a period of 2 weeks before the crossover, at which time the collection schedule was repeated.

Figure 2. Urine volume collection. A) Pooled urine output for reach rat (ml) at the end of each session. Data presented as individual sample values and within-subjects comparison for each session. *p<0.01. B) Urine collected from the LabSand sessions only. Urine was collected every half hour and the volume determined. Data is presented as the mean ± SEM of the cumulative volume for each subject across each session.

Figure 3. Stress indicators. A) Each animal was weighed at the beginning and the end of each session and the difference calculated as weight lost during the session. B) Fecal pellets were counted for each animal at the end of each session. C) Corticosterone concentration

was determined by ELISA for each animal's pooled urine sample for each session. For A-C, all data presented as individual sample values and within-subjects comparison for each session. *p<0.01. D-E) Representative images of ambulatory behavior during the collection sessions – metabolic cage (D) and LabSand (E). F-G) Representative images of sleeping behavior during the collection sessions – metabolic cage (D) and LabSand (E).

Tables

Urinalysis Measurement	Collection Method	Session				
		2 hr	4 hr	6 hr (1)	6 hr (2)	6 hr (3)
Refractive Index	MC	1.343 *(0.003)*	1.343 *(0.003)*	1.342 *(1.343)*	1.346 *(0.004)*	1.344 *(0.003)*
	LS	1.340 *(0.007)*	1.342 *(0.004)*	1.343 *(0.003)*	1.344 *(0.005)*	1.344 *(0.004)*
Specific Gravity	MC	1.028 *(0.011)*	1.030 *(0.009)*	1.026 *(0.006)*	1.039 *(0.011)*	1.033 *(0.010)*
	LS	1.026 *(0.007)*	1.029 *(0.012)*	1.030 *(0.006)*	1.035 *(0.013)*	1.032 *(0.011)*
Leukocytes (cacells/µl)	MC	90.63 *(40.92)*	123.8 *(154.8)*	83.75 *(48.75)*	135.6 *(153.3)*	81.88 *(42.84)*
	LS	83.75 *(48.75)*	70.00 *(41.58)*	54.38 *(50.81)*	104.4 *(40.92)*	73.13 *(51.82)*
Protein (g/l)	MC	0.78 *(0.97)*	0.74 *(0.99)*	0.37 *(0.41)*	1.06 *(1.22)*	0.58 *(0.46)*
	LS	1.42 *(1.38)*	1.45 *(1.38)*	0.74 *(0.99)*	0.81 *(0.94)*	0.56 *(0.36)*
pH	MC	8.25 *(0.38)*	8.13 *(0.44)*	8.00 *(0.38)*	8.13 *(0.52)*	7.50 *(0.00)*
	LS	8.19 *(0.26)*	8.00 *(0.53)*	8.00 *(0.27)*	7.75 *(0.46)*	7.69 *(0.37)*
Ketones (mmol/l)	MC	0.63 *(0.74)*	0.12 *(0.26)*	0.75 *(0.80)*	0.20 *(0.25)*	0.63 *(0.35)*
	LS	0.63 *(0.74)*	0.44 *(0.50)*	0.75 *(0.80)*	0.46 *(0.48)*	0.50 *(0.46)*
Creatinine	MC	0.63 *(0.23)*	0.58 *(0.19)*	0.64 *(0.27)*	0.55 *(0.26)*	0.56 *(0.27)*
	LS	0.59 *(0.15)*	0.59 *(0.36)*	0.68 *(0.23)*	0.74 *(0.26)*	0.57 *(0.24)*

Table 1. Analysis of common, clinically relevant urine markers or properties. Data presented as mean *(SD)* for each group (collection method and individual session) and analyzed by paired within-subjects t-test within each collection session. Refractive index and specific gravity were determined with a refractometer. Leukocytes, protein, pH, and ketone levels were determined with URS-10T test strips. Creatinine was determined by colorimetric kit. All values are in normal range for rats. No comparisons were significantly different.

toxics

Article

Hydrophobic sand is a non-toxic method of urine collection, appropriate for urinary metal analysis in the rat

Jessica F. Hoffman[1,*], Vernieda B. Vergara[1], Steven R. Mog[2], and John F. Kalinich[1]

1 Internal Contamination and Metal Toxicity Program, Armed Forces Radiobiology Research Institute, Uniformed Services University, Bethesda, MD
2 Office of Food Additive Safety (OFAS), CFSAN/FDA/DHHS, College Park, MD
* Correspondence: jessica.hoffman@usuhs.edu; Tel.: 301-295-9118

Academic Editor: name
Received: date; Accepted: date; Published: date

Abstract: Hydrophobic sand is a relatively new method of urine collection in the rodent, comparable to the established method using a metabolic cage. Urine samples are often used in rodent research, especially for biomarkers of health changes after internal contamination from embedded metals, such as in a model of a military shrapnel wound. However, little research has been done on the potential interference of hydrophobic sand with urine metal concentrations either by contamination from the sand particulate, or adsorption of metals from the urine. We compare urine collected from rats using the metabolic cage method and the hydrophobic sand method for differences in metal concentration of common urinary metals, and examine physical properties of the sand material for potential sources of contamination. We found minimal risk of internal contamination of the rat by hydrophobic sand, and no interference of the sand with several common metals of interest (cobalt, strontium, copper, and manganese), although we advise caution in studies of aluminum in urine.

Keywords: urine, metal contamination, internal contamination, rodent

Abbreviations

DU	Depleted uranium
AFRRI	Armed Forces Radiobiology Research Institute
LS	LabSand
MC	Metabolic cage
ICP-MS	Inductively coupled plasma mass spectroscopy

1. Introduction

The development of the full metal-jacketed bullet around the time of the Spanish-American War in 1898 improved survivability from battle wounds and increased the probability of embedded metal fragments in survivors [1]. Embedded metal fragments were initially considered inert, and a low health risk, until the appearance of several case reports on medical issues associated with embedded fragment wounds suffered during wartime many years prior to manifestation of the adverse health effect [2-7].

The majority of research into health effects of embedded metals has been conducted in the context of the safety of implanted devices [8], with little focus on long-term health effects of military-relevant metals and metal mixtures [9] until several U.S. military personnel were wounded by depleted uranium (DU) fragments during Operation Desert Storm in 1991. Standard medical protocol was to leave fragments in place for the life of the individual. However, due to DU's chemical and radiological properties and little information available on the long-term health effects of embedded DU, the need for research into the biokinetics and toxicology of DU became clear. The Armed Forces Radiobiology Research Institute (AFRRI) developed and validated a rodent model system to assess the health effects of embedded metal fragments [10]. Results of this investigation led to a reassessment of the Department of Defense (DoD) fragment removal policy for DU, recommending excising fragments larger than 1 cm in diameter and patients be followed for any long-term adverse health effects [11].

The concern over DU embedded fragment health effects led to the search for replacement materials for DU munitions. Several tungsten-based compositions were then tested for adverse health effects using the AFRRI embedded fragment model system, but it was discovered that the tungsten/nickel/cobalt composition induced malignant, highly aggressive rhabdomyosarcomas at

the implantation sites [12], while a tungsten/nickel/iron composition did not result in any tumor formation [13,14]. Underscoring our current lack of knowledge regarding long-term health effects of military-relevant metal fragments is the high number of military personnel returning wounded from the recent conflicts in Iraq and Afghanistan. Between multiple munition types, vehicle armor, and improvised explosive devices (IEDs), the list of metals and metal mixtures that may potentially be found as embedded fragments is extensive. As a result, the DoD and the Department of Veterans Affairs (DVA) have developed a list of "metals of concern" with respect to embedded fragments [15], but the biokinetic and toxicological properties of many of these metals when embedded as fragments are not yet known.

In an effort to address these problems, our ongoing research projects investigate biokinetic, toxicological, and carcinogenic effects of several military-relevant metals by using the implanted metal rodent system and examining changes in urine, serum, and tissue samples. Rodent urine is commonly collected through the use of metabolic cages, which can be stressful for the animals [16,17] and requires habituation [18]. An alternate method of rodent urine collection, hydrophobic sand, has recently come to the market. Originally developed for urine collection in the cat, hydrophobic sand is a biodegradable material with a non-toxic hydrophobic coating that causes urine to pool on its surface, making it easy to collect. The material is currently available as "LabSand" to the scientific community, or "Kit4Cat" commercially. A review of both products' safety data sheets [19,20], as well as telephone communication with the supplier (Coastline Global, Inc., Palo Alto, CA), indicate they are identical. If urine is to be assayed for biomarkers and dissolved metals in our embedded metal fragment model system, it is imperative to know whether the hydrophobic sand could contaminate urine samples with extraneous metals or adsorb baseline metals from urine. Previously we compared metabolic cage and hydrophobic sand urine collection methods for stress and clinical markers and found no significant differences that would compromise normal urine markers [21]. Here, we used the same urine samples from that experimental set to determine if there is a difference in urine metal concentration between the two collection methods. Further, we thoroughly examined the physical properties of LabSand and Kit4Cat to discover if hydrophobic sand could adsorb metals from urine, or leech out any metals and contaminate urine samples through contact with urine before collection, or from being ingested by the rat.

2. Materials and Methods

The animals, urine collection methods, experimental design, and urine samples are the same as those reported in Hoffman et al 2017. These methods are repeated here in brief. All other methods

described afterward are unique to this work.

2.1. Animals

Male Sprague Dawley rats (Envigo, Frederick, MD) were maintained on a 12:12 light:dark cycle with access to food and water *ad libitum*. Rats were pair-housed except during urine collection periods. Rats underwent no treatment or experimental conditions beyond exposure to both urine collection methods. All procedures involving animals were approved by the AFRRI Institutional Animal Care and Use Committee under protocol 2016-05-006.

2.2. Urine collection methods

2.2.1. Metabolic cages

Animals were in a standard circular metabolic cage with a wire mesh floor with urine collected in a Nalgene tube at the bottom of a funnel system. Urine could only be collected at the end of the session.

2.2.2. Hydrophobic sand

Animals were in a rectangular microisolator cage with the sand lining the bottom of the cage in place of regular bedding; urine pools on top of the sand, which is then collected with a pipette. For each rat, we collected urine every half hour and was subsequently pooled at the end of the session. The pooled urine sample for each animal was used for analysis in the current report.

2.3. Experimental design for urine collection

The experimental design and urine sample collection schedule is illustrated in Figure 1 of Hoffman et al 2017. We used a within-subjects crossover design where rats were randomly assigned to two groups: (A) was the metabolic cage followed by LabSand, (B) was LabSand followed by the metabolic cage, n=4 for each group for a total of 8 animals in both collection methods, serving as their own control. Both groups were run simultaneously under the same testing conditions. There were a total of 5 collection sessions (a 2 hour, a 4 hour, and three separate 6 hour sessions), each separated by a rest period of at least 48 hours. The method crossover occurred after the last session and the entire pattern was repeated. Food and water were not provided to any animal during urine collection sessions, but each animal was provided with a water replacement gel in a plastic cup (HydroGel®, Clear H_2O, Westbrook, ME). The gel material can be eaten by the rat for hydration but does not drip and dilute urine samples as a water bottle could.

2.4. Creatinine concentration in urine.

Creatinine concentrations in urine collected during metabolic cage and LabSand sessions were reported in Table 1 of Hoffman et al 2017, and subsequently used to normalize metal concentrations reported here. Creatinine concentrations were also determined for urine collected from the bladder of all 8 rats after euthanasia using the same assay as before. Briefly, a colorimetric creatinine assay kit (Oxford Biomedical Research, Inc., Oxford, MI) was used to determine the difference in absorbance wavelength after picric acid is added to urine, then again after addition of an acid reagent. Values were compared against a creatinine standard curve, all absorbance values were read on a spectrophotometer (SpectraMax 190, SoftMax Pro 2.0 software, Molecular Devices, Sunnyvale, CA).

2.5. Examining potential internalization of hydrophobic sand by rats

One month after completion of the metabolic cage versus LabSand experiment, rats were humanely euthanized by isoflurane exposure followed by exsanguination and confirmatory pneumothorax and lung and gut tissues collected to be examined for evidence of inhalation or ingestion of sand particulate. Of the 8 rats that had gone through the metabolic cage / LabSand crossover method experiment, 3 rats were placed in cages with LabSand 2 hours prior to euthanasia ("Acute Exposure"), and the other 3 rats were left in their home cage for the same period before euthanasia ("Past Exposure," equating to 1 month between last sand exposure and euthanasia). The stomach was opened and physically examined for any evidence of ingestion of hydrophobic sand. Additionally, lung tissue from 3 naïve rats ("No Exposure") never exposed to hydrophobic sand were collected.

All lung tissue was fixed in 10% buffered formalin, processed and embedded in paraffin, sectioned in 5-6 μm thick slices onto glass slides, and stained with hematoxylin and eosin (HE) stain for histology by the AFRRI pathology division. Slides were then examined by a board-certified veterinary pathologist using a BX51 Olympus microscope at 100X magnification under both bright

light and simple polarizing light using a U-ANT analyzer and a U-POT polarizer. Representative photomicrographs were chosen to demonstrate an arteriole, terminal bronchiole, and a larger bronchiole to show the internal positive control for birefringence (normal supportive fibrous connective tissue-collagen around the vessel and major airway).

2.6. Assessment of cytotoxicity

2.6.1. Cell line and media

V79 Chinese hamster lung fibroblasts were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and maintained in Dulbecco's Modified Eagle's Medium (D-MEM, Invitrogen, Grand Island, NY) with 10% fetal bovine serum (FBS, Invitrogen) at 37°C in a humidified atmosphere of 5% CO_2 in air. Cells were passed twice per week and were used between passages 5 and 12.

2.6.2. Cell treatment

LabSand (1g) was mixed in 10 ml of D-MEM with 10% FBS for 24h at room temperature by gentle mixing on a nutator. The mixture was then centrifuged at 400 x g for 10 min at room temperature and the supernatant filtered through a 0.2 μm to create an extraction solution. The extraction solution was then tested at full strength (undiluted, "100% Extract"), diluted 1:10 with D-MEM with 10%FBS ("10% Extract"), or diluted 1:100 with D-MEM with 10% FBS ("1% Extract"). Cells were plated on 96-well tissue culture plates at a predetermined concentration for maximum response in a toxicity assay (described below). In replicates of 6, cells were incubated for 24 hours in the following groups: Control (D-MEM with 10% FBS media only), 1% Extract, 10% Extract, or 100% Extract. After the 24 hour incubation cells were assayed for viability.

2.6.3. Viability assay

Metabolic viability (MTT assay) was assessed using the CellTiter 96® Aqueous One Solution Cell Proliferation Assay kit (Promega Corporation, Madison, WI). The assay for metabolic viability is based upon the ability of dehydrogenase enzyme systems, located in the cell mitochondria, to reduce a tetrazolium compound to a colored formazan product, which is easily detected colorimetrically. Briefly, one hour prior to the termination of the 24 hr treatment incubation period, 10 μl of CellTiter 96® Aqueous One Solution Reagent was added to each well of the plate and the plate returned to the incubator for 1 hr. After this time, the absorbance was determined at 490 nm using a microplate reader (SpectraMax Model 250 Microplate Spectrophotometer, Molecular Devices Corporation, Sunnyvale, CA). Metabolic viability of the extract-treated cells was normalized to the media-only control cells.

2.7. Analyses of physical properties of LabSand

2.7.1. Determination of hydrophobic sand particle size

5 g of LabSand or Kit4Cat brands of hydrophobic sand was placed in a Scienceware Mini-sieve Micro Sieve Set (Bel-Art Products, Wayne, NJ) using the following mesh screen sizes: 25, 35, 45, and 60 standard mesh (0.71, 0.50, 0.35, and 0.25 mm, respectfully). The apparatus was gently shaken by hand for 5 min, then each of the various fractions were weighed and normalized to the total weight of the recovered fractions in 3 separate replications.

2.7.2. Imaging of hydrophobic sand particles

A small sample of LabSand was placed on a slide and examined at 2X under bright light on an Olympus BX61 microscope using an Olympus DP72 camera (Olympus America, Inc., Center Valley, PA).

2.8. Metal analysis by ICP-MS

All compounds used in this study were obtained from Sigma-Aldrich Co. (St. Louis, MO) or Thermo Fisher Scientific (Pittsburgh, PA) and were of the highest grade available. Plastic ware and other disposables were also obtained from Thermo Fisher Scientific.

Samples were first analyzed using survey scans across the full atomic mass range of likely metal analytes via ICP-MS. Metals observed at higher amounts than the control were identified for further quantification, as were analytes that displayed larger-than-expected peaks. ICP-MS operating conditions and parameters can be found in Table S1. Limit of Detection (LoD) / Limit of Quantitation (LoQ), in ppb, are as follows: Al – 0.38/0.44; Co – 0.03/0.06; Cu – 0.24/0.54; Pb – 0.02/0.04; Sr – 0.01/0.05; Zn – 2.80/3.01; Fe – 1.08/1.85.

2.8.1. Urine metal analysis

Urine samples from metabolic cage, LabSand, and bladder collections described above were diluted in 2% nitric acid and measured by ICP-MS. Samples were then normalized against creatinine (mg/ml) to give a ng metal / mg creatinine value.

2.8.2. Analysis of metal recovery from hydrophobic sand

In order to assess whether metals in a sample could nonspecifically bind to LabSand, solutions of various metals including Al, Co, Cu, Pb, Sr, and Zn were mixed with LabSand (0.1 g) for various times. Samples were centrifuged (13,000 x g for 10 min at room temperature) and the resulting supernatant removed and analyzed for metal content using ICP-MS. Recovery of metals in contact with LabSand for 5, 15, or 60 min were compared to control.

2.8.3. Digestion of hydrophobic sand by synthetic rat gut fluid and nitric acid

Approximately 0.1 g of LabSand was treated with 1.0 ml of simulated gastric fluid by mixing on a nutator for 2 h at room temperature. Simulated gastric fluid was prepared according to Ansoborlo et al (1999) [22]. The extraction mixture was centrifuged content using ICP-MS in survey scan mode followed by quantitation of those metals present. Similarly, at 13,000 x g for 10 min at room temperature and the resulting supernatant removed and analyzed for metal a 70% nitric acid (Optima Grade, Fisher Scientific, Pittsburgh, PA) solution was used to determine maximum metal that could be digested out of the hydrophobic sand.

2.8.4. Metal analysis of HydroGel®

To determine metal content in the HydroGel® hydration cup gel samples, approximately 0.1 g of gel were cut from the HydroGel® and placed in tared glass vials and mass determined. Nitric acid (5 ml of 70% Optima Grade) was added and the gel allowed to dissolve overnight at room temperature. Aliquots of the dissolved gel were analyzed for metal content via ICP-MS in survey scan mode followed by quantitation of those metals present.

2.8.5. Treatment of metabolic cage pieces with simulated urine

Components of the metabolic cage apparatus that were in extended contact with urine during the collection procedure were assessed for removable copper contamination using the following procedure. Simulated urine solution was prepared following the method of Issacson (1968) [23]. Metabolic cage pieces (collection ring, funnel, collection cylinder) were washed with a laboratory detergent (Contrex, DeCon Labs, King of Prussia, PA) and rinsed extensively with tap water. One group of metabolic cage components were allowed to air dry, while the second set was further washed with deionized water (18 mΩ, Elga Purelab Water System, Highwycombe, Bucks, United Kingdom) before air drying. The metabolic cage components were then treated with the simulated urine solution. The collection ring and collection tubes were filled with 5 ml of simulated urine solution for 2h at room temperature. The simulated urine fluid was then collected. For the metabolic

cage funnel, 5 ml of simulated urine solution was passed through the funnel 5 times before collecting. Aliquots of the collected simulated urine solution were analyzed for copper content using ICP-MS.

2.9. Statistical analysis

For cell cytotoxicity, the percent change from control for each extract was compared to 100% (not toxic) using a one sample t-test. Extracts were then compared to each other using a one-way ANOVA. Animal urine metal concentrations were analyzed as a within-subjects two-tailed t-test comparison between collection methods for each session time. Urine collected from the bladder at the time of animal euthanasia was used as a control for urinary metal concentration in a one-way ANOVA within-subjects comparison with the third 6-hour session metabolic cage and LabSand urine samples. Metals after gastric solution or nitric solution digestion were compared via t-test. Particle size, aluminum, and strontium distribution by fractions were each analyzed by two-way ANOVA, followed by a post-hoc Sikak's multiple comparisons test if there was a main effect between fractions. Changes in metal concentration from a spiked metal standard mixed with LabSand for 5, 15, or 60 minutes were analyzed by subtracting the 0 time metal concentration from each time point and compared to 0 PPB (no change) using a one-sample t-test. All analyses used GraphPad Prism Software (version 7.01, La Jolla, CA). *P* values less than 0.05 were considered significant.

3. Results

3.1. Risk of internalization and cytotoxicity

If hydrophobic sand is to be used as a method of urine collection in rodents, it is important to determine whether the animals exposed to hydrophobic sand are at risk of internalization either through inhalation of small particles or ingestion of the material, posing a potential health risk and/or source of contamination of subsequent urine and tissue samples. One month after the conclusion of the metabolic cage vs LabSand experiment in Hoffman et al 2017, all 8 rats were euthanized and their stomach contents examined for presence of any sand grain particles. Three rats were placed in a cage with hydrophobic sand for 2 hours prior to euthanasia. No grains of hydrophobic sand were found in any stomach contents. Additionally, lung tissue was collected from 9 total animals: 3 naïve control rats that never underwent the metabolic cage vs LabSand experiment and thus were never exposed to hydrophobic sand (deemed the "Never Exposed" group), 3 rats that were part of the experiment but were not reintroduced to hydrophobic sand prior to euthanasia (the "Past Exposure" group), and 3 rats that were part of the experiment but also reintroduced to the hydrophobic sand for 2 hours prior to euthanasia (the "Acute Exposure" group). HE stained lung tissues from all 9 rats were examined microscopically under both bright light and polarized light, which would highlight any significant silica particulate foreign material trapped in the tissue. All lung tissues were normal, with no silicate crystals identified and no significant aggregates of any inflammatory cells around terminal airways in any group (Figure 1, A-F).

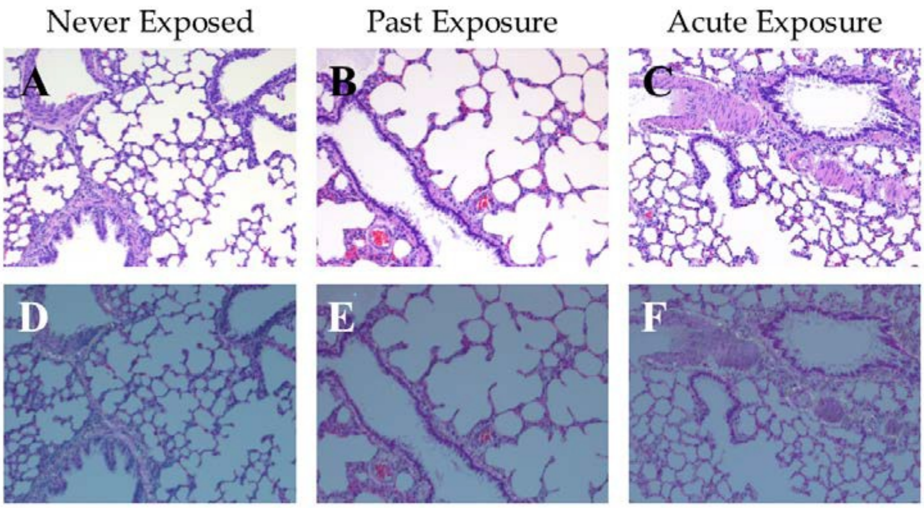
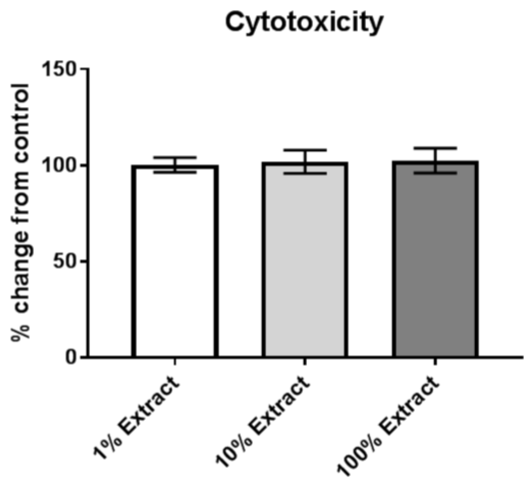


Figure 1. Representative photos of rat lung tissue at 100X; sections show an inflated area that included an arteriole, terminal bronchiole, and a larger bronchiole. (A-C) HE stained tissue under bright light microscopy, and (D-F) corresponding HE stained tissue under polarized light. (A and D) tissue from a naïve rat that were never exposed to hydrophobic sand ("Never Exposed"). (B and E) tissue from a rat that underwent all LabSand experimental sessions, and was euthanized 1 month after the conclusion of the experiment with no further exposure to hydrophobic sand ("Past Exposure"). (C and F) tissue from a rat that underwent all LabSand experimental but was also placed in a cage with hydrophobic sand for 2 hours prior to euthanasia ("Acute Exposure").

Next we wanted to determine if anything toxic to cells could leech off hydrophobic sand and pose a health risk if a rat was to ingest or inhale particulate. LabSand was agitated gently in cell media on a nutator for 24 hours, then the media filtered of all particulate to create an "extract." V79 Chinese hamster lung fibroblast cells were plated onto 96-well plates and treated with normal media or a dilution of the filtered media mixed with LabSand ("1% Extract" is a 1:100 dilution, "10% Extract" is a 1:10 dilution, and "100% Extract" is undiluted extract media). After 24 hours of exposure to the various concentrations of LabSand-exposed media, cells were evaluated for survival using a metabolic viability (MTT) assay and calculated as percent change from the normal media control where 100% indicates no change from control. The media extracts are not significantly different from each other (one-way ANOVA, $F_{2,15}=0.262$, $p=0.77$), nor is any dilution's percent change from control significantly different from 100% (one-sample t-tests: 1% Extract, $t_5=0.089$, $p=0.93$; 10% Extract,



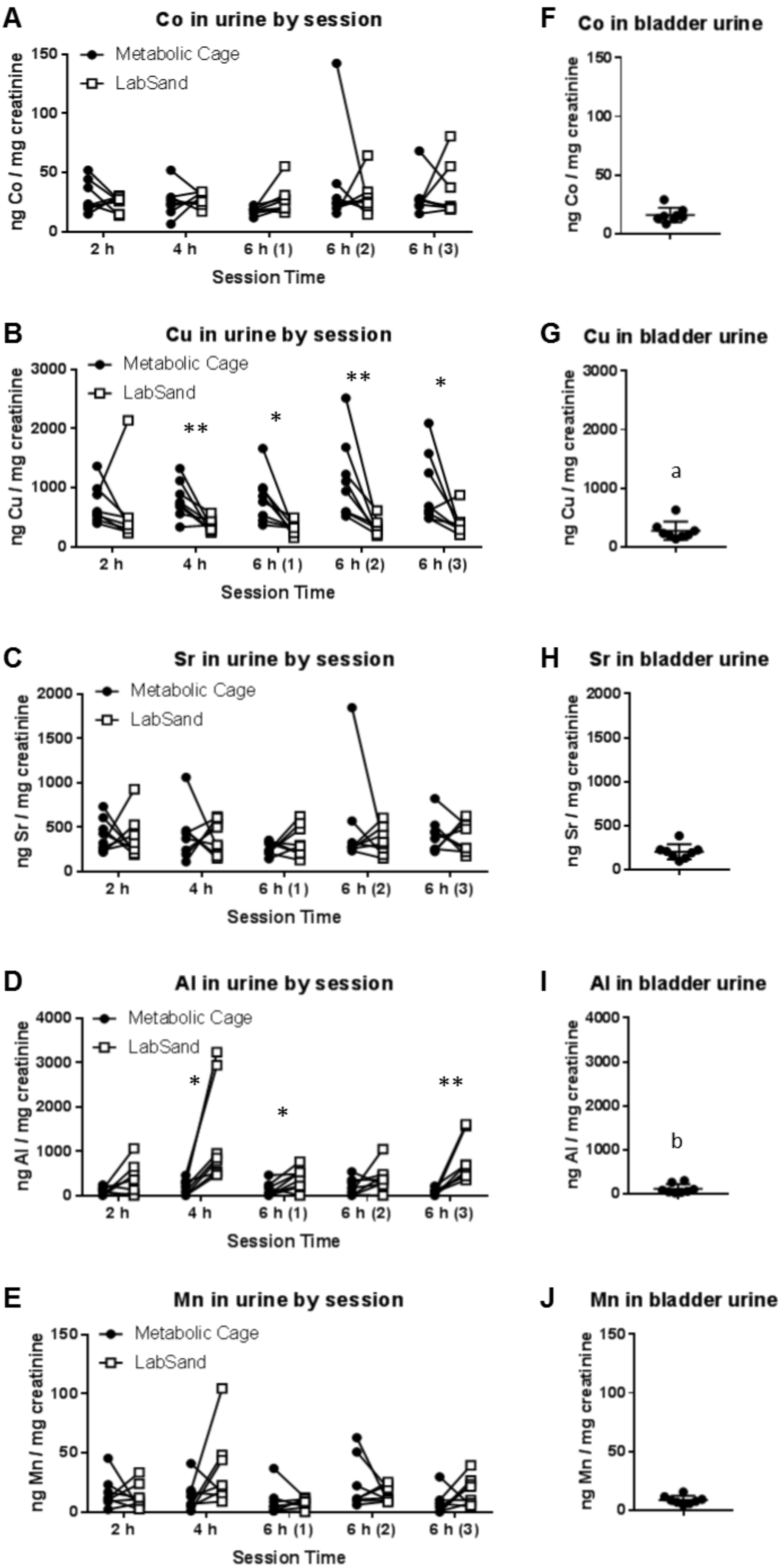
$t_5=0.712$, $p=0.51$; 100% Extract, $t_5=0.906$, $p=0.41$) (Figure 2).

Figure 2. Metabolic viability (MTT) assay of V79 Chinese hamster lung fibroblast cells exposed to varying concentrations of media that has been mixed with hydrophobic sand.

3.2. LabSand vs Metabolic Cage: metal in urine

Past research into the effects of an embedded fragment model on metal concentrations in urine has used metabolic cages for rodent urine collection. Hoffman et al 2017 suggests hydrophobic sand is a useful alternative urine collection method, but we need to ensure there is no metal contamination of urine samples from the hydrophobic sand before moving forward with this collection method. To do this, we scanned the urine samples from the metabolic cage versus LabSand experiment, as well as urine collected from the bladder at euthanasia, for cobalt (Co), copper (Cu), strontium (Sr), aluminum (Al), manganese (Mn), zinc (Zn), lead (Pb), and uranium (U) using ICP-MS and normalized to creatinine for each sample. Zn, Pb, and U concentrations were below the detectible limit, but the rest of the metals were compared within each session time using a within-subjects t-test (metabolic cage vs LabSand collection method). Urine collected from the bladder was never in contact with hydrophobic sand or the metabolic cage equipment, so this was used as a control for urinary metal concentration in a one-way ANOVA within-subjects comparison with the third 6-hour session metabolic cage and LabSand urine samples.

For cobalt, there were no significant differences in urine concentration between metabolic cage and LabSand urine collection methods in any collection session (2h session, t_7=0.922, p=0.39; 4h session, t_7=0.311, p=0.76; 6hr-1, t_7=2.251, p=0.06; 6hr-2, t_7=0.576, p=0.58; 6h-3, t_7=0.516, p=0.62) (Figure 3A). There were also no significant differences between bladder urine or the 6-hour metabolic cage or LabSand urines ($F_{(4.5,10.3)}$=2.864, p=0.112) (Figure 3F). For all time points except the 2-hour session, copper in urine from the LabSand collection method is lower than copper in urine from the metabolic


A
Co in urine by session
Metabolic Cage
LabSand
ng Co / mg creatinine
Session Time
2 h
4 h
6 h (1)
6 h (2)
6 h (3)
F
Co in bladder urine
B
Cu in urine by session
ng Cu / mg creatinine
G
Cu in bladder urine
a
C
Sr in urine by session
ng Sr / mg creatinine
H
Sr in bladder urine
D
Al in urine by session
ng Al / mg creatinine
I
Al in bladder urine
b
E
Mn in urine by session
ng Mn / mg creatinine
J
Mn in bladder urine

307

Figure 3. Metal concentrations in urine comparing metabolic cage and LabSand collection methods (A-E) and urine collected from the bladder (F-J). Asterisks indicate significant differences in urine metal concentration between collection methods for that session (*$p<0.05$, **$p<0.01$), (a) denotes a significant difference in metal concentration between bladder urine and metabolic cage urine from session 6h-3, and (b) denotes a significant difference in metal concentration between bladder urine and LabSand urine from session 6h-3.

cage method (2h session, t_7=0.630, p=0.55; 4h session, t_7=3.505, **$p<0.01$; 6hr-1, t_7=3.235, *$p<0.05$; 6hr-2, t_7=3.513, **$p<0.01$; 6h-3, t_7=2.435, *$p<0.05$) (Figure 3B). This would suggest LabSand is somehow absorbing copper out of the urine that pools on it. However, when bladder urine concentrations were compared to the third 6-hour session methods ($F_{(1.2,8.4)}$=7.534, *$p<0.05$), we found that copper in the LabSand urine is not significantly different from copper in the bladder urine (Tukey's multiple comparison test, p=0.530), but rather copper in the metabolic cage urine is surprisingly higher than copper in the bladder urine (Tukey's, $^{a}p<0.05$) (Figure 3G).

For strontium, there were no significant differences in urine concentration between metabolic cage and LabSand urine collection methods in any collection session (2h session, t_7=0.209, p=0.84; 4h session, t_7=0.152, p=0.88; 6hr-1, t_7=1.223, p=0.26; 6hr-2, t_7=0.775, p=0.46; 6h-3, t_7=0.280, p=0.79) (Figure 3C). There were also no significant differences between bladder urine or the 6-hour metabolic cage or LabSand urines ($F_{(1.7,11.9)}$=3.79, p=0.06) (Figure 3F). Aluminum is significantly higher in urine from the LabSand collection method compared with the metabolic cage collection method in the 4-hour (t_7=3.105, *$p<0.05$), first 6-hour (t_7=2.88, $p<0.05$), and third 6-hour (t_7=3.952, **$p<0.01$) sessions, but not the 2-hour (t_7=1.925, p=0.096) or second 6-hour (t_7=1.561, p=0.162) sessions (Figure 3D). When bladder urine concentrations were compared to the third 6-hour session methods ($F_{(1.0,7.2)}$=14.06, **$p<0.01$), we found that aluminum in the metabolic cage urine is not significantly different from aluminum in the bladder urine (Tukey's multiple comparison test, p=0.355), but is higher in the LabSand urine in the bladder urine (Tukey's, $^{b}p<0.05$) (Figure 3I).

For manganese, there were no significant differences in urine concentration between metabolic cage and LabSand urine collection methods in any collection session (2h session, t_7=0.492, p=0.638; 4h session, t_7=1.724, p=0.128; 6hr-1, t_7=0.332, p=0.75; 6hr-2, t_7=1.01, p=0.346; 6h-3, t_7=1.497, p=0.178) (Figure 3E). There were also no significant differences between bladder urine or the 6-hour metabolic cage or LabSand urines ($F_{(1.5,10.6)}$=2.082, p=0.177) (Figure 3J).

3.3. Potential sources of contamination

Significantly higher levels of aluminum in urine from the LabSand urine samples compared to bladder samples suggests aluminum may be leeching out of the hydrophobic sand to contaminate urine, but significantly higher levels of copper in urine from the metabolic cage urine samples compared to bladder samples without any difference between LabSand and bladder urine concentrations suggest metal contamination of urine is not necessarily straightforward contamination by LabSand. To investigate potential sources of metal contamination in urine samples, we conducted several further tests with LabSand and metals. First we wanted to determine what metals were present, and in what concentrations, in LabSand brand hydrophobic sand under maximum digestive conditions, and what concentrations may be extracted out of that sand if they were to be ingested by a rat. Approximately 0.1 g samples of LabSand were mixed with either a synthetic gastric fluid solution or 70% nitric acid solution (for maximum metal extraction) for 2 hours, which is the normal transit time through the rat stomach [24]. Samples were filtered and measured for metal via ICP-MS. In the survey scan, only aluminum and strontium appeared in any quantity above control, and were subsequently quantitated. Significantly less aluminum (mean: 111.1, SD: 33.79) was able to be extracted from the LabSand digested in synthetic gastric fluid than from LabSand digested in 70% nitric acid (mean: 1140, SD: 208.8 ng/g sand; t_4=8.42, **$p<0.01$) (Figure 4A), which represents the maximum amount of aluminum that could be extracted from the LabSand. Strontium levels after extraction from LabSand digested with synthetic gastric fluid were below the

limits of detection, and levels were very low when digested with the maximum condition, 70% nitric acid mean: 37.23, SD: 31.40 ng/g sand) (**Figure 4B**).

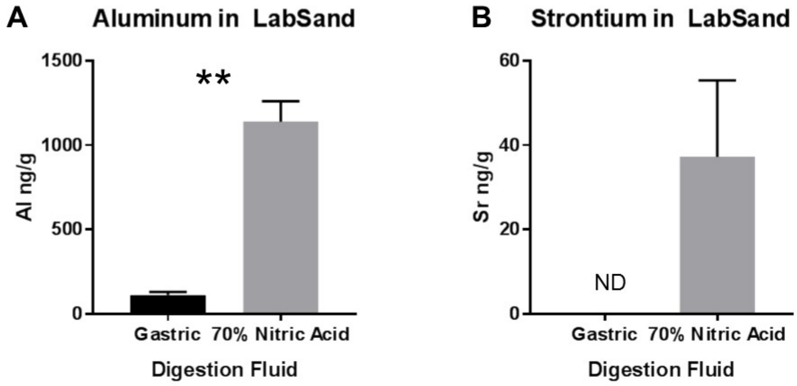


Figure 4. Digestion of LabSand brand hydrophobic sand in either synthetic gastric fluid or 70% nitric acid. (A) Aluminum and (B) Strontium extracted from LabSand under the two digestive conditions. **p<0.01, ND = not detected.

Next we wanted to know the particle size distribution of hydrophobic sand, as well as any potential differences in metal concentrations within that particle size distribution. We examined both LabSand and Kit4Cat brands, which the manufacturer lists as nearly identical. Visual inspection of both hydrophobic sand brands under brightfield at 2X magnification reveal a similar distribution of particles of varying sizes, from large pebble-like structures to fine dust (Figure 5A-B). A sieve system was then used to separate LabSand or Kit4Cat particulate into 5 fractions according to size and each fraction was calculated as a percent of the total weight, then compared as distribution across fractions within a brand as well as between brands within fraction by two-way ANOVA. There was a significant main effect of particle fraction distribution ($F_{4,20}=187.6$, $p<0.0001$) for both brands, but no difference between brands in the distribution across fractions ($F_{1,20}<0.001$, $p=0.99$) and no interaction effect ($F_{4,20}=0.99$, $p=0.44$) (Figure 5C). In both brands of hydrophobic sand, more than 50% of the particles were larger than 0.50 mm (LabSand, 60.3%; Kit4Cat, 63.7%) and only a small percentage is smaller than 0.25 mm (LabSand, 6.8%; Kit4Cat, 6.1%).

Fractions were then subjected to the same extraction process for metals using the 70% nitric acid solution as before and analyzed for aluminum and strontium by ICP-MS, as those were the metals above control on the survey scan for the whole sample for each brand. There was a main effect of fraction ($F_{4,20}=100.9$, $p<0.0001$) where aluminum concentration was distributed unequally between particle size fractions. For both brands, the highest concentration of aluminum occurred in the smallest fraction size (<0.25 mm; LabSand mean: 1747.66, SD:91.05 ng/g sand; Kit4Cat mean 1402.36, SD: 144.74 ng/g sand). There was also a main effect of brand ($F_{1,20}=59.7$, $p<0.0001$) but no interaction effect ($F_{4,20}=0.297$, $p=0.877$). The concentration of aluminum was significantly greater in LabSand than in Kit4Cat within every particle size fraction: <25 mm ($t_{20}=4.323$, $p<0.01$), 0.25-0.35 mm ($t_{20}=2.98$, $p<0.05$), 0.35-0.50 mm ($t_{20}=3.634$, $p<0.01$), 0.50-0.71 mm ($t_{20}=3.086$, $p<0.05$), >0.71 mm ($t_{20}=3.254$, $p<0.05$) (Figure 5D). LabSand and Kit4Cat had no significant differences in strontium concentration or distribution across particle size fractions. There was no significant main effect of particle fraction ($F_{4,20}=0.989$, $p=0.436$), brand ($F_{1,20}=2.705$, $p=0.116$), or interaction ($F_{4,20}=0.552$, $p=0.700$) (Figure 5E). The concentration of strontium in the less than 25 mm particle fraction is 61.65 (SD: 0.560) ng/g LabSand and 47.308 (SD:7.742) ng/g Kit4Cat.

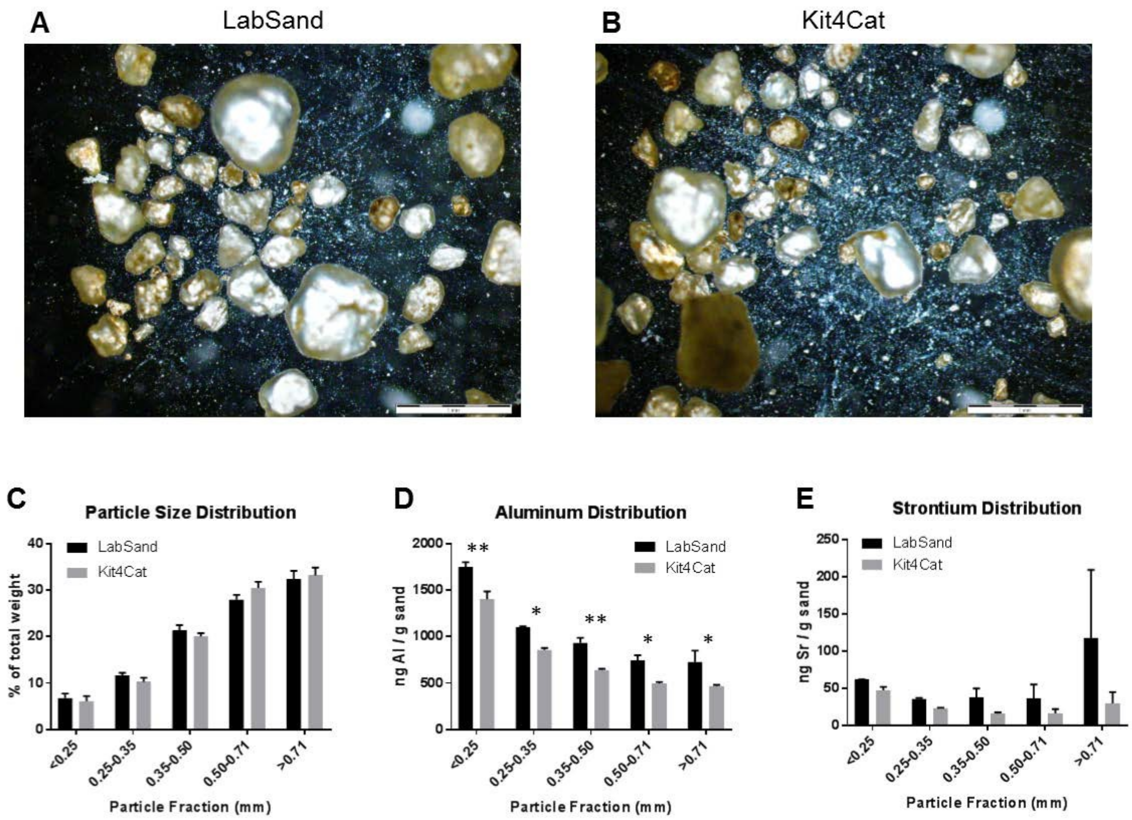


Figure 5. Distribution of particles in hydrophobic sand brands. Brightfield images under 2X magnification for (A) LabSand and (B) Kit4Cat sand samples. (C) Particle size distribution across 5 size fractions for both brands of hydrophobic sand. (D) Aluminum distribution across size fractions for both brands. (E) Strontium distribution across size fractions for both brands. $*p<0.05$, $**p<0.01$

Now that we knew aluminum and strontium were the only metals at risk of leeching out of LabSand into the animals if ingested, or potentially into the urine as contamination, the next question was whether metals in the urine might bind to the sand and be pulled from urine before it could be collected. Urine was collected every half hour, so the longest time any pool of urine was in contact with the hydrophobic sand was 30 min. We used a spiked standard for each metal of interest (Al, Sr, Cu, Co, Sr, Zn) in water and mixed (not placed on top, to replicate a worst-case scenario) with 0.1 g of LabSand for 5, 15, or 60 minutes, then filtered, measured for metal concentration, and the spiked control value was subtracted from each time point to give the change in metal concentration. These values were then compared to 0 PPB by a one-sample t-test to determine if there was a significant change from the spiked metal concentration (Table 1). We used water instead of a synthetic urine solution because proteins in synthetic urine could interact with the metals and obscure any interaction between metals and LabSand. Aluminum had no significant change in concentration from spiked control at either the 5 min (t_2=3.372, p=0.078) or 60 min (t_2=2.742, p=0.111) time points, but showed a small but statistically significant increase of 0.654 ng/ml at the 15 min time point (t_2=17.61, $**p<0.01$). Strontium had a small but statistically significant increase in concentration from spiked control at all three time points: 1.143 ng/ml at 5 min (t_2=10.33, $**p<0.01$), 0.810 ng/ml at 15 min (t_2=6.372, $*p<0.05$), and 1.090 ng/ml at 60 min (t_2=11.68, $**p<0.01$). Copper had a very small but statistically significant increase in concentration over spiked control (0.057 ng/ml) only at the 60 min time point (t_2=109.3, $**p<0.01$). Cobalt had no significant change from control at any time point (5 min, t_2=0.161, p=0.887; 15 min, t_2=1.089, p=0.390; 60 min, t_2=0.046, p=0.968).

Table 1. Changes in spiked metal concentrations after various times mixing with LabSand.

Metal	**Time spent mixing with LabSand**

	5 min	15 min	60 min
Aluminum	1.254 (*0.644*)	0.654 (*0.064*)**	2.35 (*1.484*)
Strontium	1.143 (*0.192*)**	0.810 (*0.220*)*	1.090 (*0.162*)**
Copper	-0.013 (*0.14*)	0.074 (*0.117*)	0.057 (*0.001*)**
Cobalt	0.204 (*0.136*)	0.100 (*0.180*)	-0.006 (*0.240*)
Zinc	-2.917 (0.*340*)**	-2.717 (*0.251*)**	-2.26 (*0.265*)**
Lead	0.205 (*0.015*)**	0.270 (*0.022*)**	0.189 (*0.036*)*

Values presented as mean (*SD*), with units in PPB (ng/ml). A positive mean indicates metal concentration increased over spiked standard after exposure to LabSand, while a negative mean indicates metal concentration decreased from spiked standard after exposure to LabSand. *p<0.05, **p<0.01

Other potential sources of contamination may exist beyond the hydrophobic sand itself. One such source of metal contamination could be the HydroGel® cups. We cut out 3 samples each from 3 different gels and analyzed them for metals via ICP-MS. Only aluminum appeared in the survey scan and subsequently quantified. We found a mean concentration of 2463 (SD 2486) ng Al / g hydrogel (Min: 28.9 ng/g, Median: 1200 ng/g, Max: 7058 ng/g). Additionally, since copper was higher in urine from the metabolic cage collections than either the LabSand or the bladder urine, which suggests something else is adding copper to the urine, we suspected the tap water used to rinse the metabolic cages after cleaning may be leaving residue after air-drying, so we tested all of the lab sinks and our 18Ω water supply for metal. Results are shown in Table 2. Sink 1 was the source of water used for washing the metabolic cages, though all four sink locations were surprisingly high in both copper and strontium concentrations compared to the 18Ω water supply.

Table 2. Metal concentrations in lab sink water.

Source	Metal concentration, PPB (ng/ml)				
	Co	Cu	Sr	Al	Mn
18Ω	-0.020	0.065	0.190	0.560	-0.010
Sink 1	0.095	*413.150*	*270.950*	5.610	3.380
Sink 2	0.180	*1411.000*	*238.700*	5.950	3.170
Sink 3	0.100	*303.950*	*258.400*	3.635	7.780
Sink 4	0.315	*105.550*	*179.200*	0.995	4.225

To follow up our discovery of high metals in the sink tap water, we ran a short experiment with the collection apparatus of the metabolic cage where we washed the collection pieces (collection ring, funnel, collection cylinder) with Contrex and allowed to air dry as was done in the original experiment, or we included an additional rinse step with deionized water, then used a synthetic urine solution to simulate a collection period of 2 hours before measuring for copper by ICP-MS. Copper concentrations (mean, SD) are presented as PPB (ng/ml) in Table 3. There was no significant difference between the way the collection pieces were rinsed (two-way ANOVA, $F_{1,12}$=0.7551, p=0.402), though the artificial urine picked up lower levels of copper levels than from either the ring or collection container ($F_{2,12}$=7.292, p<0.01).

Table 3. Copper in artificial urine after sitting on metabolic cage collection parts.

Treatment	Copper concentration, PPB (ng/ml)		
	Ring	Funnel	Container
Millipore Rinse	0.616 (*0.459*)	0.153 (*0.050*)	0.934 (*0.538*)
Tap Water alone	0.520 (*0.211*)	0.100 (*0.061*)	0.694 (*0.218*)

Values presented as mean (*SD*).

4. Discussion

Recently we have shown a relatively new method of rodent urine collection using hydrophobic sand (brand names LabSand or Kit4Cat) to be as efficient as using the metabolic cage collection method for short-term volume collections with no significant changes to clinically-relevant urinary markers or properties [21]. In order to use the hydrophobic sand collection method for future work examining urine samples in a rodent model of metal shrapnel wounds, we also wanted to ensure that the period of exposure to hydrophobic sand did not present an ingestion or inhalation risk, alter natural background metal concentrations in urine, nor contaminate urine samples with extraneous metals from the sand. To accomplish this we examined urine samples from the Hoffman et al (2017) study for changes in baseline urine metal concentrations between the metabolic cage and LabSand collection methods, as well as compared collection samples to urine collected from the bladder after euthanasia. Additionally, the stomach contents of all animals were examined for evidence of ingestion of sand, and lungs examined for evidence of inhalation of sand. We found no evidence of sand in the stomach contents of any of the 8 rats in our study, including three rats that were placed in LabSand for a two hour period directly prior to euthanasia. Similarly, a study using Kit4Cat hydrophobic sand found only 2 grains of sand in the stomach of 1 out of 10 mice [25], suggesting rodents do not typically ingest the sand material. Further, we found no evidence of sand particulate in the lung tissue of the rats in our study whether they were exposed to LabSand during only the collection periods or exposed to an additional two hours of sand directly prior to euthanasia. Comparing the lung tissue to naïve rats that were never exposed to hydrophobic sand at all, we also conclude from the histopathology that there was no inflammatory response or tissue damage from any exposure to the sand. If, however, sand was accidently ingested or inhaled by a rodent, we also determined the sand would pose a minimal risk of toxicity to tissue because there was no effect of increasing exposures of LabSand in media on the metabolic viability of Chinese hamster lung fibroblast cell cultures.

Next, by comparing background metal concentrations in urine collected using both the metabolic cage and hydrophobic sand methods, we found no significant differences between the method of collection for cobalt, strontium, or manganese concentration. Copper concentrations were lower in urine collected using the hydrophobic sand method than metabolic cage for 4 out of 5 session times. We thought this was due to adsorption of copper into the LabSand material, but comparing both methods' urine samples to urine collected from the bladder, which never had direct contact with either the metabolic cage apparatus or LabSand material, we found that copper is in fact higher in urine collected from the metabolic cage samples, and copper concentrations are not different between LabSand and the bladder samples. Metabolic cage parts are made of Nalgene, and do not contain intrinsic copper in the material. In examining the sources of water used to wash the cage parts, however, we discovered high copper concentrations in the tap water compared to purified water, which suggests droplets dried after washing deposited small amounts of copper onto the sides of the collection materials that were then picked up in the urine as it flowed down into the collection cup. To further confirm lack of contamination of copper concentrations from the hydrophobic sand, we showed that copper was not found in LabSand material after digestion by nitric acid or an artificial gastric juice solution, and there was no change in copper concentration from a spiked standard after 5, 15, or 60 minutes of mixing with LabSand material.

Aluminum was the only other metal that had any significant difference in urine concentration between the metabolic cage and LabSand collection methods, with it being higher in LabSand urine samples in 3 out of 5 collection sessions. Comparison with bladder urine concentration revealed that aluminum was, in fact, higher in the LabSand collection samples, with no difference from the metabolic cage collected samples. Nitric acid digestion of LabSand material revealed high concentrations of aluminum in the sand particulate, although internal contamination of the rat is of minimal risk – artificial gastric juice pulled significantly less aluminum out of the LabSand material after digestion. Aluminum had the highest concentration in the smallest particle fraction of both

brands of hydrophobic sand, potentially posing a source of internal contamination if inhaled, but since the smallest particle fraction also makes up the lowest percentage of fraction sizes and lack of evidence of sand particulate in the lung, increased concentration of aluminum in urine from internalization of hydrophobic sand is highly unlikely, and thus could come from contact with the sand material itself. A 15 minute period of mixing an aluminum spiked solution with LabSand did result in a significant increase of 0.654 ng/ml, but this is nowhere near enough to account for the difference of several hundred to several thousand ng/ml of aluminum in urine from the LabSand collection method over the metabolic cage collection method, especially since urine was collected from the LabSand surface every half hour. One other potential source of aluminum was the HydroGel ® water replacement material provided to each rat. We found very high levels of aluminum in some of the gel samples, which could have contaminated the urine either through direct contact, though that was rare, or ingestion of the gel material increased urine concentration temporarily. However, it is difficult to make that determination because we did not measure intake of gel for each animal, and animals in the metabolic cages did have access to gel cups from the same lot as the animals in LabSand collection sessions, and would require further, more precise study to determine the true level of contamination risk.

We conclude that the use of hydrophobic sand is an acceptable alternative method to the traditional metabolic cage method for urine collection in the rodent, especially for short-term (6 hours or less) collection periods when total urine recovery is not necessary. For most metals of interest we examined (cobalt, strontium, copper, and manganese), hydrophobic sand has no effect on background natural urinary metals, nor does it appear to adsorb or otherwise contaminate metal concentration in urine. Aluminum urine concentration, however, may be confounded by the use of hydrophobic sand, and analyses of aluminum in urine should be done with caution. However, we believe the contamination risk would be greatly minimized by immediate collection of urine pools from the hydrophobic sand surface and not using the HydroGel® material as a source of water replacement, as it has high concentrations of aluminum itself.

Supplementary Materials: The following are available online at www.mdpi.com/link, Table S1: ICP-MS operating conditions and parameters.

Acknowledgments: The work presented here was supported by the grant Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments (W81XWH-16-2-0058) from the Congressionally Directed Medical Research Programs (CDMRP) Peer Reviewed Medical Research Program. All procedures involving animals were (a) conducted with maximum possible well-being of the rats, (b) approved by the AFRRI Institutional Animal Care and Use Committee prior to the start of the study under protocol 2016-05-006, and (c) performed in compliance with the guidelines set forth in the Guide for the Care and Use of Laboratory Animals in a facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

The use of LabSand or Kit4Cat brands of hydrophobic sand in this work does not represent an endorsement of the product or company by the U.S. Government. The views expressed in the paper are those of the authors and do not reflect the official policy or position of the Armed Forces Radiobiology Research Institute, the Uniformed Services University, the Department of Defense, or the United States Government.

Author Contributions: J.K. and J.H. conceived and designed the experiments; J.H, J.K., and V.V. performed the experiments; J.H. analyzed the data; S.M. analyzed the lung histopathology, J.H. wrote the paper, all authors edited the paper.

Conflicts of Interest: The authors declare no conflict of interest. The funding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

References

1. Dougherty, P.J., Eidt, H.C. Wound ballistics: Minie ball vs. full metal jacketed bullets – a comparison of Civil War and Spanish-American War firearms. *Military Medicine*, **2009**, 174, 403-407, PMID19485111.

2. Schenck, N.L., Kronman, B.S. Hoarseness and mass in the neck 30 years after penetrating shrapnel injury. *Annals of Otology, Rhinology, and Laryngology*, **1977**, 86, 259, PMID 848838.

3. Knox, J., Wilkinson, A. Shrapnel presenting with symptoms 62 years after wounding. *British Medical Journal (Clinical Research Edition)*, **1981**, 283, 193, PMID 6789962.

4. Symonds, R.P., Mackay. C., Morley, P. The late effect of grenade fragments. *Journal of the Royal Army Medical Corps*, **1985**, 131, 68-69, PMID 4045803.

5. Lindeman, G., McKay, M.J., Taubman, K.L., Bilous, A.M. Malignant fibrous histiocytoma developing in bone 44 years after shrapnel trauma. *Cancer*, **1990**, 66, 2229-2232, PMID 2171751.

6. Ligtenstein, D.A., Krijnen, J.L.M., Jansen, B.R.H., Eulderink, F. Forgotten injury: a late benign complication of an unremoved shrapnel fragment – case report. *Journal of Trauma*, **1994**, 36, 580-582, PMID 8158725.

7. Eylon, S., Mosheiff, R., Liebergall, M., Wolf, E., Brocke, L., Peyser, A. Delayed reaction to shrapnel retained in soft tissue. *Injury: International Journal for Care of the Injured*, **2005**, 36-275-281, PMID 15664592.

8. IARC Monograph on the Evaluation of Carcinogenic Risk to Humans. Volume 74. Surgical implants and other foreign bodies. 1999, IARC Lyon, France pp 113-229.

9. Kane, M.A., Kasper, C.E., Kalinich, J.F. Protocol for the assessment of potential health effects from embedded metal fragments. *Military Medicine*, **2009**, 174, 265-269, PMID 19354090.

10. Castro, C.A., Benson, K.A., Bogo, V., Daxon, E.G., Hogan, J.B., Jacocks, H.M., Landauer, M.R., McBride, S.A., Shehata, C.W. Establishment of an animal model to evaluate the biological effects of intramuscularly embedded depleted uranium fragments. Armed Forces Radiobiology Research Institute, Bethesda, MD, Technical Report 96-3, **1996**.

11. OTSG/MEDCOM. (2011). Medical management of Army personnel exposed to depleted uranium (DU). OTSG/MEDCOM Policy Memo 11-047. Availble online: www.pdhealth.mil/downloads/OTSG_MEDCOM_Policy_11-047_Med_M.pdf Accessed September 2, 2015.

12. Kalinich, J.F., Emond, C.A., Dalton, T.K., Mog, S.R., Colman, G.D., Kordell, J.E., Miller, A.C., McClain, D.E. Embedded weapons-grade tungsten alloy shrapnel rapidly induces metastatic high-grade rhabdomyosarcomas in F344 rats. *Environmental Health Perspectives*, **2005**, 113, 729-734, PMID 15929896.

13. Schuster, B.E., Roszell, L.E., Murr, L.E., Ramirez, D.A., Demaree, J.D., Klotz, B.R., Rosencrance, A.B., Dennis, W.E., Bao, W., Perkins, E.J., Dillman, J.F., Bannon, D.I.. In vivo corrosion, tumor outcome, and microarray gene expression for two types of muscle-implanted tungsten alloys. *Toxicology and Applied Pharmacology*, **2012**, 265, 128-138, PMID 22982072.

14. Emond, C.A., Vergara, V.B., Lombardini, E.D., Mog, S.R., Kalinich, J.F. Induction of rhabdomyosarcoma by embedded military-grade tungsten/nickel/cobalt not by tungsten/nickel/iron in the $B6C3F_1$ mouse. *International Journal of Toxicology*, **2015**, 34, 44-54, PMID 25544565.

15. Health Affairs Policy Letter 07-029 (2007). Analysis of Metal Fragments Removed from Department of Defense Personnel. Available online www.health.mil/~/media/MHS/Policy%20Files/Import/07-029.ashx Accessed September 2, 2015.

16. Gil, M.C., Aguirre, J.A., Lemoine, A.P., Segura, E.T., Barontini, M., Armando, I. Influence of age on stress responses to metabolic cage housing in rats. *Cellular and Molecular Neurobiology*, **1999**, 19: 625-633, PMID 10384260.

17. Eriksson, E., Royo, F., Lyberg, K., Carlsson, H-E., Hau, J. Effect of metabolic cage housing on immunoglobin A and corticosterone excretion in faeces and urine of young rats. *Experimental Physiology*, **2004**, 89: 427-433, PMID 15131075.

18. Kurien, B.T., Everds, N.E., Scofield, R.H. Experimental animal urine collection; a review. *Laboratory Animals*, **2004**, 38: 333-361, PMID 15479549.

19. LabSand MSDS. Available online www.labsand.com/wp-content/uploads/2016/09/MSDS-LabSand.pdf Accessed March 1, 2017.

20. Kit4Cat MSDS. Available online www.kit4cat.com/wp-content/uploads/2011/04/MSDS-Kkit4Ccat.pdf Accessed March 1, 2017.

21. Hoffman, J.F., Fan, A.X., Neuendorf, E.H., Vergara, V.B., Kalinich, J.F. Hydrophobic Sand versus metabolic cages: a comparison of urine collection methods for the rat (*Rattus norvegicus*). *Journal of the American Association for Laboratory Animal Science*, **2017**. (in press)

22. Ansoborlo, E., Henge-Napoli, M.H., Chazel, V., Gibert, R., and Guilmette, R.A.. Review and critical analysis of available *in vitro* dissolution tests. *Health Physics*, **1999**, 77: 638-645, PMID 10568542.

23. Issacson, L.C. Urinary ionic strength, osmolality, and specific conductivity. *Investigative Urology*, **1968**, **5**: 406-413.

24. Enck, P., Merlin, V., Erckenbright, J.F., and Wienbeck, M. Stress effects on gastrointestinal transit in the rat. *Gut*, **1989**, 30: 455-459, PMID 2714679.

25. 3Rs Refinement – Use of hydrophobic sand in collection of analytical urine samples. Poster by Smith JC, Lancaster H, Ridley D, McClure F, Whelan G. Available at http://labsand.com/wp-content/uploads/2015/05/GSK-poster.pdf Accessed May 26, 2017.

Table S1. ICP-MS operating conditions and parameters.

ICP-MS operating conditions and parameters	
Instrument Parameters	
Nebulizer type	Concentric
Spray chamber	Conical, with impact bead
Sampler cone	Nickel, 1mm orifice diameter
Skimmer cone	Nickel, 0.7 mm orifice diameter
Sample uptake rate	1.0 ml/min
Sample read delay	45 sec
Plasma conditions	
RF power	1400 W
Plasma argon gas flow	13.0 L/min
Auxiliary argon gas flow	0.80 L/min
Nebulizer gas flow	0.91 L/min
Mass spectrometer settings	
Scanning mode	Peak jump
Sweeps	100
Dwell time	500 μs
Channels/mass	1
Acquisition time	10 sec
Number of readings/replicate	3
Number of replicates	2

APPENDICES

PROJECTS 3 & 4:
<u>Joanna Gaitens, Ph.D., MSN/MPH, RN, Project Leader/Principal Investigator, Project 3</u>
"Biomarker assessment of kidney injury from metal exposure in embedded fragment registry veterans"
<u>Stella Hines, M.D., MSPH, Project Leader/Principal Investigator, Project 4</u>
"Respiratory health in a cohort of embedded fragment registry veterans exposed to blasts and metals"

1. **Questionnaire:** "Self-Reported Health Effects in Veterans with Blast and Embedded Metal Fragment Injuries" (Study Population #1-Questionnaire Only Group)
   Project Title: "Respiratory Health in a Cohort of Embedded Fragment Registry Veterans Exposed to Blasts and Metals"

2. **Questionnaire:** ""Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments" (Study Population #2-Clinical Assessment Group)
   Project Title: "Biomarker Assessment of Kidney Injury from Metal Exposure in Embedded Fragment Registry Veterans"

3. Projects 3 and 4 Regulatory Approval Schedule

4. List of Approved Study Documents:

    1. Stamped Informed Consent
    2. HIPAA Authorization
    3. ACOS/R & D Review
    4. ISO/PO Approvals from both VA Central and local VA R & D
    5. Recruitment Letters
    6. Telephone Scripts
    7. Questionnaires
    8. Respiratory Protocols
    9. Spot Urine Collection Protocol
    10. VA Central LSI Applications (for each site)
    11. VA Central PI New Investigator Application

Study ID:________________
For use in HRPO Log No. A-19735.2 (McDiarmid- "Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments")

Self-reported Health Effects in Veterans with Blast and Embedded Metal Fragment Injuries

INSTRUCTIONS
- Use a black/blue pen.
- Do not make any stray marks on this form.
- Please answer every question as honestly as possible and to the best of your ability, unless you are requested to skip over a question. The questionnaire will take between 20-30 minutes to complete.
- Please feel free to reference any records you may have in your possession.

Section A: Basic Information

Participant ID:		Date Form completed: ____________ MM/DD/ YYYY	Gender: ☐ M ☐ F	Current Age: ________

1. Marital status:	☐ Married ☐ Separated	☐ Widowed ☐ Divorced	☐ Never married

2. Are you Spanish, Hispanic or Latino?	☐ No, not Spanish, Hispanic, Latino ☐ Yes, Mexican, Mexican American, Chicano ☐ Yes, Puerto Rican ☐ Yes, Cuban ☐ Yes, other Spanish, Hispanic, Latino
3. What is your race?	☐ White ☐ Black/ African America ☐ Chinese ☐ Japanese ☐ Asian Indian ☐ Other Asian ☐ Filipino ☐ Pacific Islander ☐ American Indian/ Alaskan Native ☐ Other:____________________
4. What is the highest degree or level of school you have completed?	☐ Less than high school ☐ High school diploma/ GED ☐ Some college credit, but no degree ☐ Associate's degree (e.g., AA, AS) ☐ Bachelor's degree (e.g., BA,BS) ☐ Master's degree (e.g., MA, MS, MBA) ☐ Professional or Doctorate degree

5. Including yourself, how many people currently live in your household?	☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7 ☐ 8 ☐ 9+
6. Which income category represents the total income of your household from all sources (before taxes and deductions) during the last 12 months?	☐ Less than $10,000 ☐ $10,000 - $19,999 ☐ $20,000 - $29,999 ☐ $30,000 - $39,999 ☐ $40,000 - $49,999 ☐ $50,000 - $59,999 ☐ $60,000 - $74,999 ☐ $75,000 - $99,999 ☐ $100,000 - $149,999 ☐ $150,000 or more ☐ Prefer not to answer

Section B: Uniformed Service Experience		
7. In which branch of the service did you serve?	☐ Army ☐ Navy ☐ Air Force ☐ Marine Corps ☐ Coast Guard	☐ National Guard ☐ Merchant Marines ☐ NOAA ☐ Public Health Service
8. At the time of your injury, please indicate if you were:	☐ Active Duty	☐ Reserves
9. Did you deploy in support of the 1990-91 Gulf War?	☐ Yes	☐ No
10. Were you ever exposed to chemical or biological warfare agents?	☐ Yes ☐ No	☐ Unsure

The following set of questions are related to blast experience that will help us assess the significance of the blast or explosion.

Section C: Blast/Injury History

11. Did you have any injury(ies) during your deployment from any of the following? (check all that apply):	☐ Fragment ☐ Bullet ☐ Vehicular (any type of vehicle, including airplane) ☐ Fall ☐ Blast (Improvised Explosive Device, RPG, Land mine, Grenade, etc) ☐ Other:______________________________	
12. Following a blast or explosion, did you experience any of the following? (check all that apply):	☐ Being dazed, confused or "seeing stars" ☐ Not remembering the injury ☐ Losing consciousness (knocked out) for less than a minute ☐ Losing consciousness for 1-20 minutes	☐ Losing consciousness for longer than 20 minutes ☐ Having any symptoms of concussion afterward ☐ Head Injury ☐ None of the above ☐ Not applicable
13. Are you currently experiencing any of the following problems that you think might be related to a possible head injury or concussion? (check all that apply):	☐ Headaches ☐ Dizziness ☐ Memory Problems ☐ Balance Problems	☐ Ringing in the ears ☐ Irritability ☐ Sleep problems ☐ Other:______________ ☐ Not applicable
14. As the result of a blast or explosion, did you experience any of the following? (check all that apply)	☐ Pneumothorax (collapsed lung) ☐ Lung contusion (bruised lung) ☐ Rib fracture (broken rib) ☐ Penetrating lung injury (gunshot wound or shrapnel to the chest) ☐ Ruptured ear drum ☐ Pain around the cheek bones, above your eyes, or in your teeth ☐ Nose bleed ☐ Sinus pressure ☐ Not applicable	

15. Did your injury require surgery?	☐ Yes	☐ No
16. Did your injury require amputation?	☐ Yes	☐ No
16a. If so, describe:	________________________________	
17. Immediately following your injury, did you notice blood in your urine?	☐ Yes ☐ No	☐ Unsure
18. Have you ever been told you had a traumatic brain injury (TBI) by a physician?	☐ Yes	☐ No

The following set of questions will allow us to 1.) describe health conditions that may be associated with retained fragments and 2.) identify other sources of metal exposure.

Section D: Fragment and Metal Exposure Questions	
19. In what year did you have an injury that led to having an embedded fragment? (if more than one, enter the year of the first injury)	__________ Year
20. Location when you received the injury that resulted in shrapnel or fragments being removed from or remaining in your body:	☐ Afghanistan ☐ Iraq ☐ Other
The next several questions ask about your embedded fragment injury.	
21. Were you injured by a bullet? ☐ Yes ☐ No	

22. Were you injured as a result of a blast or explosion? ☐ Yes ☐ No

If no, skip to question #25.

22. a. If yes, approximately how many meters were you from the explosion?
_______________m

23. Were you in a vehicle at the time of the blast or explosion? ☐ Yes ☐ No

24. Was the blast or explosion caused by (check all that apply):

☐ Improvised Explosive Device (IED)

☐ Rocket Propelled grenade

☐ Land mine

☐ Grenade

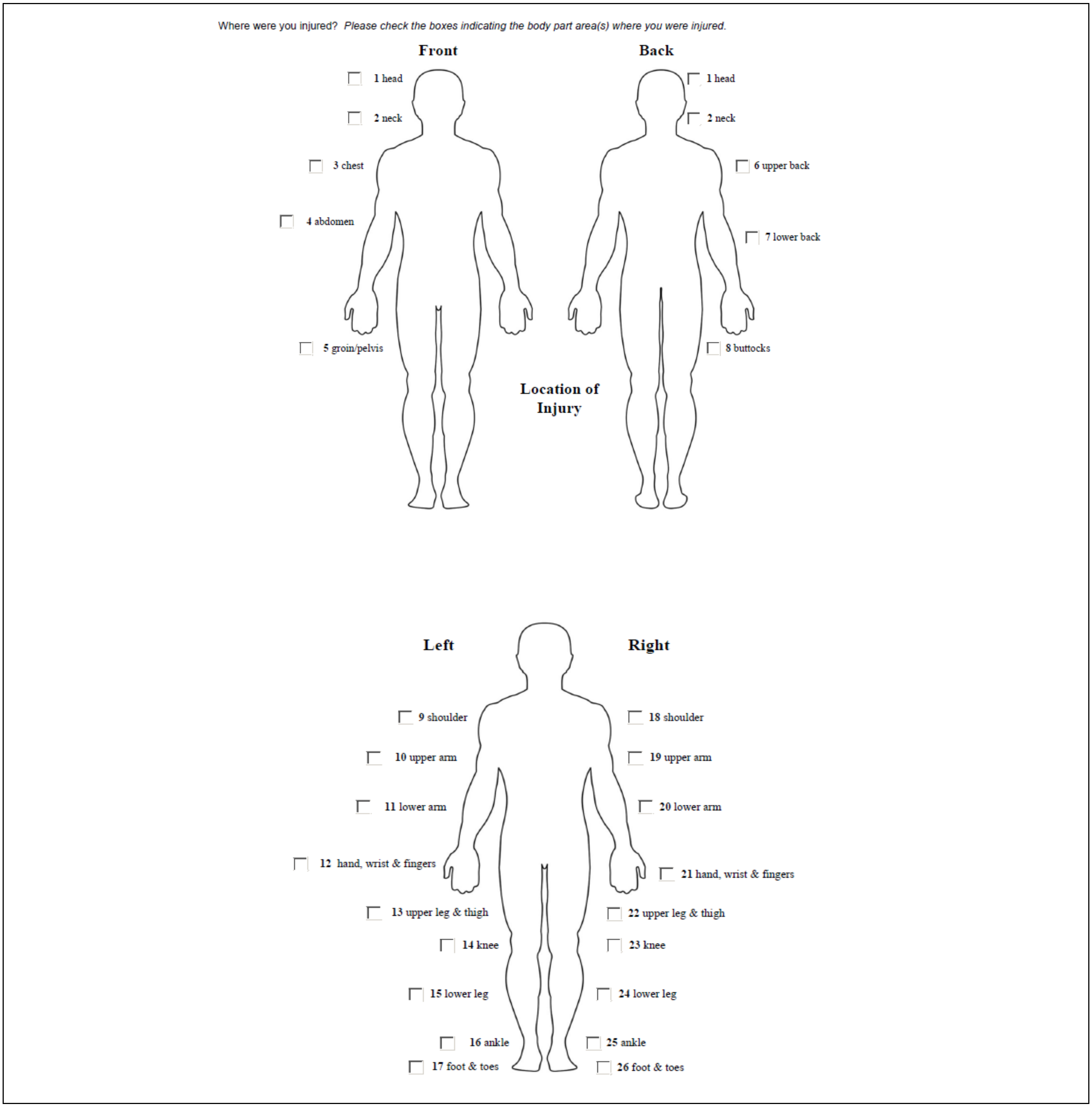
☐ Enemy fire

☐ Friendly fire

☐ Unknown

☐ Other, please describe: ______________________

25. Where were you injured? Please check the boxes indicating the body part area(s) where you were injured.

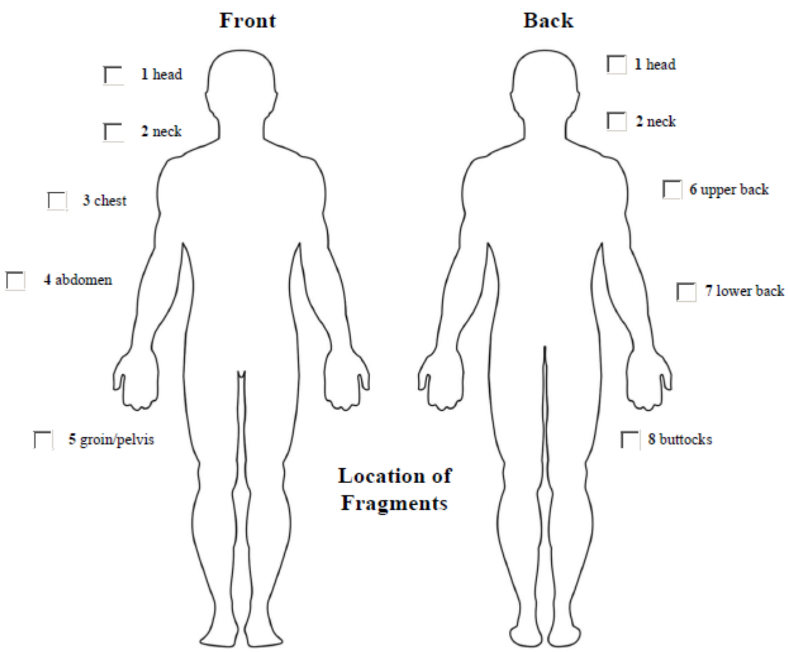


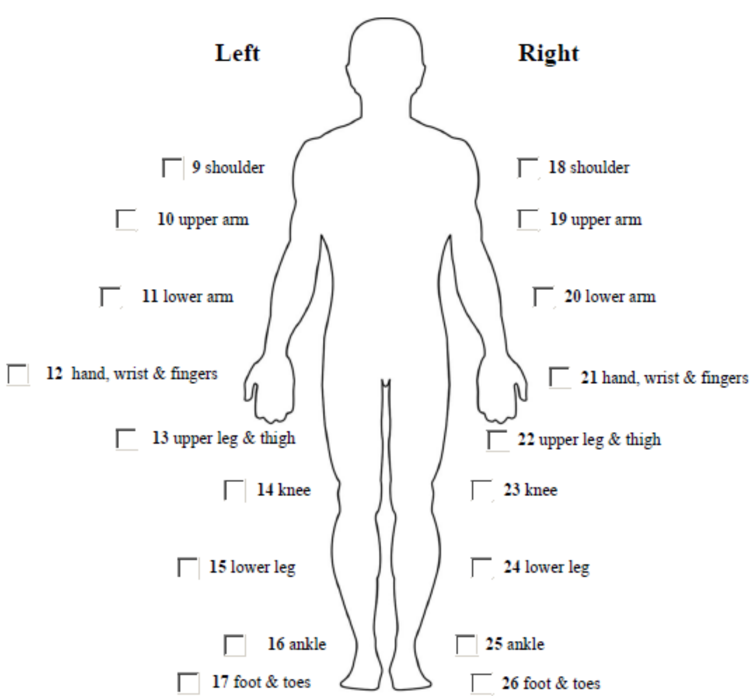
26. Did you have shrapnel, fragments, or bullets removed during surgery? ☐ Yes ☐ No ☐ Unknown 26.b… If yes, were the fragments sent to the lab for analysis? ☐ Yes ☐ No ☐ Unknown
27. Do you have retained fragments or shrapnel in your body from bullets or a blast or explosion? ☐ Yes ☐ No ☐ Unknown 27.b… If yes, where? *Please check the boxes indicating the body part area(s) where the fragments are located.* *(continued on next page)*

Study ID:________________

For use in HRPO Log No. A-19735.2 (McDiarmid- "Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments")

*Please **check the boxes** indicating the body part area(s) where **fragments** are located.*





28. Where were you treated for this injury?

- ☐ In the field
- ☐ At a Combat Support Hospital
- ☐ At Landstuhl, Germany
- ☐ At a U.S. based Medical Treatment Facility
- ☐ At a VA Medical Center

The next several questions ask about other sources of metal exposure.

29. In the past year, have you worked in an occupation or had a hobby that involved the following?

(check all that apply)

- ☐ Smelting
- ☐ Demolition
- ☐ Mining
- ☐ Soldering
- ☐ Welding
- ☐ Machining, grinding of metals
- ☐ Sand blasting
- ☐ Other manufacturing that involves working with metals
- ☐ Making bullets or shot
- ☐ Firing range use or maintenance
- ☐ Working with wood preservatives
- ☐ Making stained glass
- ☐ Making fishing weights
- ☐ Working with anti-foulant (marine) paint
- ☐ Working with lead paint
- ☐ Making jewelry or art using metals
- ☐ I have not worked in an occupation or hobby that involved any of the above during the past year

30a. In the past year, have you worked in an occupation in which you were exposed to metal dust or fumes in any other way?

☐ Yes ☐ No

If yes, please describe:

____

____

30b. In the past year, have you had a hobby in which you were exposed to metal dust or fumes in any other way?

☐ Yes ☐ No

If yes, please describe:

____

____

31. Do you currently have any of the following? (check all that apply)	☐ Metal braces on your teeth ☐ Tattoos ☐ Piercings ☐ I do not have any of the above.

32. Do you have any of the following implants/ devices in your body?

☐ Hip, knee or shoulder replacement	____________ *Year Implanted*	____________ *Location in Body*
☐ Surgical Clips or wires	____________ *Year Implanted*	____________ *Location in Body*
☐ Metal plates, screws or rods	____________ *Year Implanted*	____________ *Location in Body*
☐ Stents	____________ *Year Implanted*	____________ *Location in Body*
☐ Pacemaker or defibrillator	____________ *Year Implanted*	____________ *Location in Body*
☐ Dental implants	____________ *Year Implanted*	____________ *Location in Body*
☐ Other: ____________	____________ *Year Implanted*	____________ *Location in Body*

33. Do you routinely use/take the following? (check all that apply)

- ☐ Vitamins
- ☐ Ayurvedic medicines
- ☐ Denture cream
- ☐ Nutritional or dietary supplements
- ☐ Zinc sunblock

	☐ I do not routinely use/take any of the above.
35. What is the primary source of your household water?	☐ Community Water System ☐ Well

Sometimes people have fragments in a part of their body different from the site of their injury. The following questions address both the fragment site and the injury site. Please answer accordingly.

36. How often do you experience…

36a. …skin irritation near the site of a fragment?

☐ Often ☐ Sometimes ☐ Rarely ☐ Never ☐ Unsure of fragment location

36b. …skin irritation near the site of the injury?

☐ Often ☐ Sometimes ☐ Rarely ☐ Never

36c. …pain around the site of a fragment?

☐ Often ☐ Sometimes ☐ Rarely ☐ Never ☐ Unsure of fragment location

36d. …pain around the site of the injury?

☐ Often ☐ Sometimes ☐ Rarely ☐ Never

36e. …swelling around the site of a fragment?

☐ Often ☐ Sometimes ☐ Rarely ☐ Never ☐ Unsure of fragment location

36f. …swelling around the site of the injury?

☐ Often ☐ Sometimes ☐ Rarely ☐ Never

37. Have you had fragments work their way out of your body (without surgery)? ☐ Yes ☐ No

38. Do you have any area on your skin that is discolored (i.e., darkened, tattoo-like appearance) that you believe is related to a fragment? ☐ Yes ☐ No

39. Can you feel any of the fragments under your skin? ☐ Yes ☐ No

40. Do you have a fragment located in a joint space? ☐ Yes ☐ No ☐ Unsure of fragment location

40a. If so, where: ________________

41. Have you ever broken a bone? ☐ Yes ☐ No

41. a.... If "yes", when (check all that apply)?

☐ Before fragment injury

☐ At the time of fragment injury

☐ After fragment injury

42. Have you ever been told that you have a metal allergy or sensitivity? ☐ Yes ☐ No

42a. If "yes", to which metal? ______________________________

43. Have you ever been told you have contact dermatitis? ☐ Yes ☐ No

43a. If "yes", was it believed to be related to a metal exposure? ☐ Yes ☐ No

44. Have you ever been told that you have eczema? ☐ Yes ☐ No

45. Have you ever been told you had lead poisoning? ☐ Yes ☐ No

The following set of questions will help us describe your overall health status.

Section E: General Health, Activities and Habits

46. In general, would you say your health is:

☐ Excellent ☐ Very Good ☐ Good ☐ Fair ☐ Poor

47. The following items are about activities you might do during a typical day. **Does your health now limit you in these activities? If so, how much?**

47a. Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf?

☐ Yes, limited a lot ☐ Yes, limited a little ☐ No, not limited at all

47b. Climbing several flights of stairs?

☐ Yes, limited a lot ☐ Yes, limited a little ☐ No, not limited at all

48. As a result of problems with your **physical health**, in the **last 4 weeks**, have you....

48a….accomplished less than you would like?

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

48b… been limited in the kind of work or other activities?

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

49. As a result of any **emotional problems** (such as feeling depressed or anxious), in the **last 4 weeks**, have you….

49a….accomplished less than you would like?

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

49b….not done work or other activities as carefully as usual?

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

50. During the past 4 weeks, how much did **pain** interfere with your normal work (including both work outside the home and housework)?

☐ All of the time ☐ Most of the time ☐ A good bit of the time ☐ Some of the time ☐ A little of the time ☐ None of the time

51. How much of the time **in the last 4 weeks**…..

51a….have you felt calm and peaceful?

☐ All of the time ☐ Most of the time ☐ A good bit of the time ☐ Some of the time ☐ A little of the time ☐ None of the time

51b….did you have a lot of energy?

☐ All of the time ☐ Most of the time ☐ A good bit of the time ☐ Some of the time ☐ A little of the time ☐ None of the time

51c….have you felt downhearted and blue?

☐ All of the time ☐ Most of the time ☐ A good bit of the time ☐ Some of the time ☐ A little of the time ☐ None of the time

52. **During the past 4 weeks**, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives, etc.)?

☐ All of the time ☐ Most of the time ☐ A good bit of the time ☐ Some of the time ☐ A little of the time ☐ None of the time

53. How many **prescription** medications do you currently take on a daily basis?

☐ None ☐ 1-3 ☐ 4-6 ☐ 7-9 ☐ 10 or more

54. How many **non-prescription** medications do you currently take on a daily basis?

☐ None ☐ 1-3 ☐ 4-6 ☐ 7-9 ☐ 10 or more

55. Do you take any of the following medications regularly (2 or more times a week)? (check all that apply)

☐ Aspirin
☐ Ibuprofen (Motrin)
☐ Naproxen (Aleve)
☐ Meloxicam (Mobic)
☐ Celecoxib (CeleBREX)
☐ Goody's Pain Relief Powder
☐ BC Pain Relief Powder
☐ None of the medications listed

55a. If you checked any of the above, approximately how many months have you been taking this medication regularly?

☐ <1 month ☐ 1-6 months ☐ 6-12 months ☐ 12-24 months ☐ >24 months

* Questions 46-52 were taken from The Veterans RAND 12 Item health Survey (VR-12). The VR-12 was derived from the Veterans RAND 36 Item Health Survey (VR-36) which was developed from the MOS RAND SF-36 Version 1.0. It was modified from its original version for the purposes of this study.

The following set of questions will ask you about other symptoms you may experience.

Section F: Organ-Specific Health Questions					
Rate the severity of each of the following symptoms on a scale from 0 (not at all) to 4 (extremely).					
56. Do you often notice a bad taste in your mouth?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
57. Do you experience loss of appetite?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
58. Do you often feel nauseous or sick to your stomach?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
59. Do you vomit frequently?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
60. Do you experience heart burn?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
61. Do you notice abdominal bloating or excessive gas symptoms?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
62. Do you experience diarrhea?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
63. Do you experience constipation?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
64. Did you frequently get hiccoughs ("hiccups")?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
65. Do you experience itching?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
66. Do you often develop hives or any other type of rash?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
67. Do you bruise or bleed easily?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
68. Do you experience a lack of pep or energy?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
69. Do you tire easily or experience weakness?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
70. Do you develop muscle cramps?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely

71. Do you often feel faint when you stand up?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
72. Do you find yourself having difficulty falling and/or staying asleep?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
73. Do you find yourself falling asleep during the day?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
74. Do you feel irritable often?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
75. Do you experience decreased alertness?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
76. Do you experience forgetfulness?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
77. Do you notice that your vision is blurry?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
78. Do you ever notice blood in your urine?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
79. Do you experience swelling or puffiness of the skin, particularly around your eyes?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
80. Do you find yourself getting up to urinate frequently throughout the night?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely

For the following section, please check "yes" or "no" for each item.

81. I have been **tested** for chronic kidney disease.	☐ Yes	☐ No
82. I have been **told I have** chronic kidney disease.	☐ Yes	☐ No
83. My age is:		
83a. Between 50 and 59 years of age.	☐ Yes	☐ No
83b. Between 60 and 69 years of age.	☐ Yes	☐ No
83c. 70 years of age or older.	☐ Yes	☐ No
84. I have or have had anemia.	☐ Yes	☐ No

85. I am diabetic.	☐ Yes	☐ No
86. I have a history of heart attack or stroke.	☐ Yes	☐ No
87. I have a history of congestive heart failure.	☐ Yes	☐ No
88. I have a circulation disease in my legs.	☐ Yes	☐ No
89. I have protein in my urine.	☐ Yes	☐ No
90. I have a history of high blood pressure.	☐ Yes	☐ No
91. I have a history of lupus, scleroderma or other autoimmune disease.	☐ Yes	☐ No
92. I have a history of recurrent urinary tract infection (UTI).	☐ Yes	☐ No
93. I have a history of recurrent kidney stones.	☐ Yes	☐ No
94. I have a family history of chronic kidney disease.	☐ Yes	☐ No
95. Has a doctor ever told you that you have:		
95a. hypertension (high blood pressure)	☐ Yes	☐ No
95b. cardiovascular (heart) disease	☐ Yes	☐ No
95c. kidney cancer	☐ Yes	☐ No
95d. high cholesterol	☐ Yes	☐ No
95e. an infection or inflammation of the kidneys	☐ Yes	☐ No

The following set of questions will help us assess your lung function.

Section G: Lung Function

For the following section, please check one option for each item.

96. Do you usually have a cough? (Count a cough with first smoke or on first going out of doors. Exclude clearing of throat.).

☐No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

If your answer is "No, none of the time" to the above question, check N/A to the following question.

96a. Do you usually cough as much as 4 to 6 times a day, 4 or more days out of the week?

☐ N/A ☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

97. Do you usually bring up phlegm from your chest? (Count phlegm with first smoke or first going out of doors. Exclude phlegm from nose.)

☐No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

If your answer is "No, none of the time" to the above question, check N/A to the following question.

97a. Do you usually bring up phlegm like this as much as twice a day, 4 or more days out of the week?

☐ N/A ☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

98. Does your chest ever sound wheezy or whistling…..

98a. …..when you have a cold?

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

98b.…occasionally apart from colds?

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

98c.…most days and nights?

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

99. Do you ever have attacks of wheezing that make you feel short of breath?

☐No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

If your answer is "No, none of the time" to the above question, check N/A to the following questions.

99a. How old were you when you had your first attack? ☐ N/A ________________ Age

99b. Have you had two or more such episodes? ☐ N/A ☐ Yes ☐ No

99c. Have you ever required medicine or treatment for these attacks? ☐ N/A ☐ Yes ☐ No

100. Are you troubled by shortness of breath when hurrying on the level (a flat surface) or walking up a slight hill?
☐No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

101. Do you have to walk slower than people of your age on the level (a flat surface) because of breathlessness?
☐No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

102. Do you ever have to stop for breath when walking at your own pace on the level (a flat surface)?
☐No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

103. Are you too breathless to leave the house or breathless on dressing and undressing?
☐No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

104. During the past 3 years, have you had chest illnesses that have kept you off work, indoors or in bed?
☐No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

105. Have you ever had any of the following?
105a. Bronchitis? ☐ Yes ☐ No
105b. Pneumonia? ☐ Yes ☐ No
105c. Hay fever/ seasonal allergies? ☐ Yes ☐ No

106. Have you ever had <u>chronic</u> bronchitis? ☐ Yes ☐ No
If your answer is "No" to the above question, check N/A to the following questions.
106a. Do you still have it? ☐ N/A ☐ Yes ☐ No

106b. Was it confirmed by a doctor? ☐ N/A ☐ Yes ☐ No

106c. At what age did it start? ☐ N/A ____________
Age when started

107. Have you ever had emphysema? ☐ Yes ☐ No

If your answer is "No" to the above question, check N/A to the following questions.

107a. Do you still have it? ☐ N/A ☐ Yes ☐ No

107b. Was it confirmed by a doctor? ☐ N/A ☐ Yes ☐ No

107c. At what age did it start? ☐ N/A ____________
Age when started

108. Have you ever had asthma? ☐ Yes ☐ No

If your answer is "No" to the above question, check N/A to the following questions.

108a. Do you still have it? ☐ N/A ☐ Yes ☐ No

108b. Was it confirmed by a doctor? ☐ N/A ☐ Yes ☐ No

108c. At what age did it start? ☐ N/A ____________
Age when started

108d. Do you currently require medicine or treatment for asthma? ☐ N/A ☐ Yes ☐ No

109. Have you ever had any other chest illnesses? ☐ Yes ☐ No

If "yes", please specify: ____

110. Have you <u>ever</u> had any chest injuries (check as many as apply)?
- ☐ Pneumothorax (collapsed lung)
- ☐ Lung contusion (bruised lung)
- ☐ Rib fracture (broken rib)
- ☐ Penetrating lung injury (gunshot wound or shrapnel to the chest)

111. Have you ever worked for a year or more in a dusty job? ☐ Yes ☐ No

111a. If "yes", please specify industry: ____

111b. If "yes", was dust exposure: ☐ Mild ☐ Modest ☐ Severe

112. Have you ever been exposed to gas or chemical fumes in your work? ☐ Yes ☐ No

112a. If "yes", please specify industry: ______________________________

112b. If "yes", was gas or chemical fume exposure: ☐ Mild ☐ Modest ☐ Severe

113. Have you ever smoked cigarettes (NO means less than 100 cigarettes in your lifetime)? ☐ Yes ☐ No

If your answer is "No" to the above question, check N/A to the following questions.

113a. Do you now smoke (as of one month ago)? ☐N/A ☐ Yes ☐ No

113b. At what age did you start? ☐N/A ____________ Age when **started**

113c. If you have stopped smoking cigarettes completely, how old were you when you stopped? ☐N/A ____________ Age when **quit**

113d. On average of the entire time you smoked, how many cigarettes did you smoke per day?

☐N/A ☐ 0.5-1 pack/ week ☐ 1 pack/week ☐ 1-1.5 packs/ day ☐ 1.5-2 packs/day ☐ > 2 packs/ day

114. Have you ever smoked non-tobacco products regularly (i.e. vape, e-cigarettes)? ☐ Yes ☐ No

114a. If "yes", please specify ______________________________

Assessing the Health Effects of Blast Injuries and Embedded Metal Fragments

INSTRUCTIONS

- Use a black/blue pen.
- Do not make any stray marks on this form.
- Please answer every question as honestly as possible and to the best of your ability, unless you are requested to skip over a question. The questionnaire will take between 15-20 minutes to complete.
- Please feel free to reference any records you may have in your possession.

Section A: Basic Information

Study ID:		Date Form completed: ____________ MM/DD/ YYYY	Gender: ☐ M ☐ F	DOB: ____________ MM/DD/ YYYY

1. Marital status: ☐ Married ☐ Widowed ☐ Separated ☐ Divorced ☐ Never married

2. Are you Spanish, Hispanic or Latino?
   - ☐ No, not Spanish, Hispanic, Latino
   - ☐ Yes, Mexican, Mexican American, Chicano
   - ☐ Yes, Puerto Rican
   - ☐ Yes, Cuban
   - ☐ Yes, other Spanish, Hispanic, Latino

3. What is your race?
   - ☐ White
   - ☐ Black/ African America
   - ☐ Chinese
   - ☐ Japanese
   - ☐ Asian Indian
   - ☐ Other Asian
   - ☐ Filipino
   - ☐ Pacific Islander
   - ☐ American Indian/ Alaskan Native
   - ☐ Other:____________________

4. What is the highest degree or level of school you have completed?
   - ☐ Less than high school
   - ☐ High school diploma / GED
   - ☐ Some college credit, but no degree
   - ☐ Associate's degree (e.g., AA, AS)
   - ☐ Bachelor's degree (e.g., BA, BS)
   - ☐ Master's degree (e.g., MA, MS, MBA)
   - ☐ Professional or Doctorate degree

5. Including yourself, how many people ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐ 6 ☐ 7 ☐ 8 ☐ 9+

currently live in your household?	
6. Which income category represents the total income of your household from all sources (before taxes and deductions) during the last 12 months?	☐ Less than $10,000 ☐ $10,000 - $19,999 ☐ $20,000 - $29,999 ☐ $30,000 - $39,999 ☐ $40,000 - $49,999 ☐ $50,000 - $59,999 ☐ $60,000 - $74,999 ☐ $75,000 - $99,999 ☐ $100,000 - $149,999 ☐ $150,000 or more ☐ Prefer not to answer

Section B: Uniformed Service Experience		
7. In which branch of the service did you serve?	☐ Army ☐ Navy ☐ Air Force ☐ Marine Corps ☐ Coast Guard	☐ National Guard ☐ Merchant Marines ☐ NOAA ☐ Public Health Service
8. At the time of your injury, please indicate if you were:	☐ Active Duty	☐ Reserves
9. Did you deploy in support of the 1990-91 Gulf War?	☐ Yes	☐ No
10. Were you ever exposed to chemical or biological warfare agents?	☐ Yes	☐ No ☐ Unsure

The following set of questions are related to blast experience that will help us assess the significance of the blast or explosion.

Section C: Blast/Injury History

11. Did you have any injury(ies) during your deployment from any of the following? (check all that apply):	☐ Fragment ☐ Bullet ☐ Vehicular (any type of vehicle, including airplane) ☐ Fall ☐ Blast (Improvised Explosive Device, RPG, Land mine, Grenade, etc) ☐ Other:____	
12. Following a blast or explosion, did you experience any of the following? (check all that apply):	☐ Being dazed, confused or "seeing stars" ☐ Not remembering the injury ☐ Losing consciousness (knocked out) for less than a minute ☐ Losing consciousness for 1-20 minutes	☐ Losing consciousness for longer than 20 minutes ☐ Having any symptoms of concussion afterward ☐ Head Injury ☐ None of the above ☐ Not applicable
13. Are you currently experiencing any of the following problems that you think might be related to a possible head injury or concussion? (check all that apply):	☐ Headaches ☐ Dizziness ☐ Memory Problems ☐ Balance Problems	☐ Ringing in the ears ☐ Irritability ☐ Sleep problems ☐ Other:_______________ ☐ Not applicable
14. As the result of a blast or explosion, did you experience any of the following? (check all that apply)	☐ Pneumothorax (collapsed lung) ☐ Lung contusion (bruised lung) ☐ Rib fracture (broken rib) ☐ Penetrating lung injury (gunshot wound or shrapnel to the chest) ☐ Ruptured ear drum ☐ Pain around the cheek bones, above your eyes, or in your teeth ☐ Nose bleed ☐ Sinus pressure ☐ Not applicable	

15. Did your injury require surgery?	☐ Yes	☐ No	
16. Did your injury require amputation?	☐ Yes	☐ No	
16a. If so, describe: ______________________			
17. Immediately following your injury, did you notice blood in your urine?	☐ Yes	☐ No	☐ Unsure
18. Have you ever been told you had a traumatic brain injury (TBI) by a physician?	☐ Yes	☐ No	

The following set of questions will allow us to 1.) describe health conditions that may be associated with retained fragments and 2.) identify other sources of metal exposure.

Section D: Fragment and Metal Exposure Questions

Sometimes people have fragments in a part of their body different from the site of their injury. The following questions address both the fragment site and the injury site. Please answer accordingly.

19. How often do you experience…

19a. …skin irritation near the site of a fragment?

☐ Often ☐ Sometimes ☐ Rarely ☐ Never ☐ Unsure of fragment location

19b. …skin irritation near the site of the injury?

☐ Often ☐ Sometimes ☐ Rarely ☐ Never

19c. …pain around the site of a fragment?

☐ Often ☐ Sometimes ☐ Rarely ☐ Never ☐ Unsure of fragment location

19d. …pain around the site of the injury?

☐ Often ☐ Sometimes ☐ Rarely ☐ Never

19e. …swelling around the site of a fragment?

☐ Often ☐ Sometimes ☐ Rarely ☐ Never ☐ Unsure of fragment location

19f. …swelling around the site of the injury?

☐ Often ☐ Sometimes ☐ Rarely ☐ Never

20. Have you had fragments work their way out of your body (without surgery)? ☐ Yes ☐ No

21. Do you have any area on your skin that is discolored (i.e., darkened, tattoo-like appearance) that you believe is related to a fragment? ☐ Yes ☐ No

22. Can you feel any of the fragments under your skin? ☐ Yes ☐ No

23. Do you have a fragment located in a joint space? ☐ Yes ☐ No ☐ Unsure of fragment location

23a. If so, where: ________________

24. Have you ever broken a bone?	☐ Yes ☐ No
24 a. If "yes", when (check all that apply)? ☐ Before fragment injury ☐ At the time of fragment injury ☐ After fragment injury	
25. Have you ever been told that you have a metal allergy or sensitivity?	☐ Yes ☐ No
25a. If "yes", to which metal? ________________________________	
26. Have you ever been told you have contact dermatitis?	☐ Yes ☐ No
26a. If "yes", was it believed to be related to a metal exposure?	☐ Yes ☐ No
27. Have you ever been told that you have eczema?	☐ Yes ☐ No
28. Have you ever been told you had lead poisoning?	☐ Yes ☐ No
29. Have you ever lived near an active lead smelter, battery recycling plant, or other industry likely to release lead?	☐ Yes ☐ No
30. Have you ever been actively involved in renovating a house built before 1960?	☐ Yes ☐ No
31. Have you eaten seafood within the past 24 hours?	☐ Yes ☐ No

The following set of questions will help us describe your overall health status.

Section E: General Health, Activities and Habits

32. In general, would you say your health is:

☐ Excellent ☐ Very Good ☐ Good ☐ Fair ☐ Poor

33. The following items are about activities you might do during a typical day. **Does your health now limit you in these activities? If so, how much?**

33a. Moderate activities, such as moving a table, pushing a vacuum cleaner, bowling, or playing golf?

☐ Yes, limited a lot ☐ Yes, limited a little ☐ No, not limited at all

33b. Climbing several flights of stairs?

☐ Yes, limited a lot ☐ Yes, limited a little ☐ No, not limited at all

34. As a result of problems with your **physical health**, in the **last 4 weeks**, have you….

34a.…accomplished less than you would like?

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

34b… been limited in the kind of work or other activities?

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

35. As a result of any **emotional problems** (such as feeling depressed or anxious), in the **last 4 weeks**, have you….

35a.…accomplished less than you would like?

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

35b.…not done work or other activities as carefully as usual?

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

36. During the past 4 weeks, how much did **pain** interfere with your normal work (including both work outside the home and housework)?

☐ All of the time ☐ Most of the time ☐ A good bit of the time ☐ Some of the time ☐ A little of the time ☐ None of the time

37. How much of the time **in the last 4 weeks**.....

37a....have you felt calm and peaceful?

☐ All of the time ☐ Most of the time ☐ A good bit of the time ☐ Some of the time ☐ A little of the time ☐ None of the time

37b....did you have a lot of energy?

☐ All of the time ☐ Most of the time ☐ A good bit of the time ☐ Some of the time ☐ A little of the time ☐ None of the time

37c....have you felt downhearted and blue?

☐ All of the time ☐ Most of the time ☐ A good bit of the time ☐ Some of the time ☐ A little of the time ☐ None of the time

38. **During the past 4 weeks**, how much of the time has your physical health or emotional problems interfered with your social activities (like visiting with friends, relatives, etc.)?

☐ All of the time ☐ Most of the time ☐ A good bit of the time ☐ Some of the time ☐ A little of the time ☐ None of the time

39. How many **prescription** medications do you currently take on a daily basis?

☐ None ☐ 1-3 ☐ 4-6 ☐ 7-9 ☐ 10 or more

40. How many **non-prescription** medications do you currently take on a daily basis?

☐ None ☐ 1-3 ☐ 4-6 ☐ 7-9 ☐ 10 or more

41. Do you take any of the following medications regularly (2 or more times a week)? (check all that apply)

- ☐ Aspirin
- ☐ Ibuprofen (Motrin)
- ☐ Naproxen (Aleve)
- ☐ Meloxicam (Mobic)
- ☐ Celecoxib (CeleBREX)
- ☐ Goody's Pain Relief Powder
- ☐ BC Pain Relief Powder
- ☐ None of the medications listed

41a. If you checked any of the above, approximately how many months have you been taking this medication regularly?

☐ <1 month ☐ 1-6 months ☐ 6-12 months ☐ 12-24 months ☐ >24 months

* Questions 32-38 were taken from The Veterans RAND 12 Item health Survey (VR-12). The VR-12 was derived from the Veterans RAND 36 Item Health Survey (VR-36) which was developed from the MOS RAND SF-36 Version 1.0. It was modified from its original version for the purposes of this study.

The following set of questions will ask you about other symptoms you may experience.

Section F: Organ-Specific Health Questions

Rate the severity of each of the following symptoms on a scale from 0 (not at all) to 4 (extremely).

42. Do you often notice a bad taste in your mouth?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
43. Do you experience loss of appetite?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
44. Do you often feel nauseous or sick to your stomach?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
45. Do you vomit frequently?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
46. Do you experience heart burn?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
47. Do you notice abdominal bloating or excessive gas symptoms?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
48. Do you experience diarrhea?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely

49. Do you experience constipation?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
50. Did you frequently get hiccoughs (“hiccups”)?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
51. Do you experience itching?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
52. Do you often develop hives or any other type of rash?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
53. Do you bruise or bleed easily?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
54. Do you experience a lack of pep or energy?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
55. Do you tire easily or experience weakness?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
56. Do you develop muscle cramps?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
57. Do you often feel faint when you stand up?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
58. Do you find yourself having difficulty falling and/or staying asleep?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
59. Do you find yourself falling asleep during the day?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
60. Do you feel irritable often?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
61. Do you experience decreased alertness?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
62. Do you experience forgetfulness?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
63. Do you notice that your vision is blurry?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
64. Do you ever notice blood in your	☐ 0	☐ 1	☐ 2	☐ 3	☐ 4

urine?	Not at all				Extremely
65. Do you experience swelling or puffiness of the skin, particularly around your eyes?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely
66. Do you find yourself getting up to urinate frequently throughout the night?	☐ 0 Not at all	☐ 1	☐ 2	☐ 3	☐ 4 Extremely

For the following section, please check "yes" or "no" for each item.

67. I have been **tested** for chronic kidney disease.	☐ Yes	☐ No
68. I have been **told I have** chronic kidney disease.	☐ Yes	☐ No
69. My age is:		
69a. Between 50 and 59 years of age.	☐ Yes	☐ No
69b. Between 60 and 69 years of age.	☐ Yes	☐ No
69c. 70 years of age or older.	☐ Yes	☐ No
70. I have or have had anemia.	☐ Yes	☐ No
71. I am diabetic.	☐ Yes	☐ No
72. I have a history of heart attack or stroke.	☐ Yes	☐ No
73. I have a history of congestive heart failure.	☐ Yes	☐ No
74. I have a circulation disease in my legs.	☐ Yes	☐ No
75. I have protein in my urine.	☐ Yes	☐ No
76. I have a history of high blood pressure.	☐ Yes	☐ No
77. I have a history of lupus, scleroderma or other autoimmune disease.	☐ Yes	☐ No
78. I have a history of recurrent urinary tract infection (UTI).	☐ Yes	☐ No
79. I have a history of recurrent kidney stones.	☐ Yes	☐ No
80. I have a family history of chronic kidney disease.	☐ Yes	☐ No
81. Has a doctor ever told you that you have:		
81a. hypertension (high blood pressure)	☐ Yes	☐ No
81b. cardiovascular (heart) disease	☐ Yes	☐ No

81c. kidney cancer	☐ Yes	☐ No
81d. high cholesterol	☐ Yes	☐ No
81e. an infection or inflammation of the kidneys	☐ Yes	☐ No

The following set of questions will help us assess your lung function.

Section G: Lung Function

For the following section, please check one option for each item.

82. Do you usually have a cough? (Count a cough with first smoke or on first going out of doors. Exclude clearing of throat.).

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

If your answer is "No, none of the time" to the above question, check N/A to the following question.

82a. Do you usually cough as much as 4 to 6 times a day, 4 or more days out of the week?

☐ N/A ☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

83. Do you usually bring up phlegm from your chest? (Count phlegm with first smoke or first going out of doors. Exclude phlegm from nose.)

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

If your answer is "No, none of the time" to the above question, check N/A to the following question.

83a. Do you usually bring up phlegm like this as much as twice a day, 4 or more days out of the week?

☐ N/A ☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

84. Does your chest ever sound wheezy or whistling…..

84a. …..when you have a cold?

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

84b.…occasionally apart from colds?

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

84c.…most days and nights?

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

85. Do you ever have attacks of wheezing that make you feel short of breath?

☐No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

If your answer is "No, none of the time" to the above question, check N/A to the following questions.

85a. How old were you when you had your first attack? ☐ N/A ____________ Age

85b. Have you had two or more such episodes? ☐ N/A ☐ Yes ☐ No

85c. Have you ever required medicine or treatment for these attacks? ☐ N/A ☐ Yes ☐ No

86. Are you troubled by shortness of breath when hurrying on the level (a flat surface) or walking up a slight hill?

☐No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

87. Do you have to walk slower than people of your age on the level (a flat surface) because of breathlessness?

☐No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

88. Do you ever have to stop for breath when walking at your own pace on the level (a flat surface)?

☐No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

89. Are you too breathless to leave the house or breathless on dressing and undressing?

☐No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

90. During the past 3 years, have you had chest illnesses that have kept you off work, indoors or in bed?

☐ No, none of the time ☐ Yes, a little of the time ☐ Yes, some of the time ☐ Yes, most of the time ☐ Yes, all of the time

91. Have you ever had any of the following?

91a. Bronchitis?	☐ Yes	☐ No
91b. Pneumonia?	☐ Yes	☐ No
91c. Hay fever/ seasonal allergies?	☐ Yes	☐ No

92. Have you ever had <u>chronic</u> bronchitis? ☐ Yes ☐ No

If your answer is "No" to the above question, check N/A to the following questions.

92a. Do you still have it?	☐ N/A	☐ Yes	☐ No
92b. Was it confirmed by a doctor?	☐ N/A	☐ Yes	☐ No
92c. At what age did it start?	☐ N/A	____________ Age when started	

93. Have you ever had emphysema? ☐ Yes ☐ No

If your answer is "No" to the above question, check N/A to the following questions.

93a. Do you still have it?	☐ N/A	☐ Yes	☐ No
93b. Was it confirmed by a doctor?	☐ N/A	☐ Yes	☐ No
93c. At what age did it start?	☐ N/A	____________ Age when started	

94. Have you ever had asthma? ☐ Yes ☐ No

If your answer is "No" to the above question, check N/A to the following questions.

94a. Do you still have it?	☐ N/A	☐ Yes	☐ No
94b. Was it confirmed by a doctor?	☐ N/A	☐ Yes	☐ No
94c. At what age did it start?	☐ N/A	____________ Age when started	

94d. Do you currently require medicine or treatment for asthma?	☐ N/A ☐ Yes ☐ No
95. Have you ever had any other chest illnesses? If "yes", please specify: ________________	☐ Yes ☐ No
96. Have you ever had any chest injuries (check as many as apply)?	☐ Pneumothorax (collapsed lung) ☐ Lung contusion (bruised lung) ☐ Rib fracture (broken rib) ☐ Penetrating lung injury (gunshot wound or shrapnel to the chest)
97. Have you ever worked for a year or more in a dusty job?	☐ Yes ☐ No
97a. If "yes", please specify industry:	________________
97b. If "yes", was dust exposure:	☐ Mild ☐ Modest ☐ Severe
98. Have you ever been exposed to gas or chemical fumes in your work?	☐ Yes ☐ No
98a. If "yes", please specify industry:	________________
98b. If "yes", was gas or chemical fume exposure:	☐ Mild ☐ Modest ☐ Severe
99. Have you ever smoked cigarettes (NO means less than 100 cigarettes in your lifetime)?	☐ Yes ☐ No
If your answer is "No" to the above question, check N/A to the following questions.	
99a. Do you now smoke (as of one month ago)?	☐N/A ☐ Yes ☐ No
99b. At what age did you start?	☐N/A ________ Age when **started**
99c. If you have stopped smoking cigarettes completely, how old were you when you stopped?	☐N/A ________ Age when **quit**
99d. On average of the entire time you smoked, how many cigarettes did you smoke per day?	

☐N/A	☐ 0.5-1 pack/ week	☐ 1 pack/week	☐ 1-1.5 packs/ day	☐ 1.5-2 packs/day	☐ > 2 packs/ day

100. Have you ever smoked non-tobacco products regularly (i.e. vape, e-cigarettes)? ☐ Yes ☐ No

100a. If "yes", please specify ____

Projects 3 & 4-Regulatory Approvals Schedule

VA Participant Recruitment Sites	VA Central IRB[a]	VA Central IRB-Local Site Investigator	VA Research Safety	VA Research &Development Committee	University HRPO[b]	DoD HRPO[c]
Baltimore	✓	✓	✓	✓	✓	Submitted 10/6/17
Gainesville	✓	✓	✓	✓		
Nashville	✓	✓	✓	✓		
Oklahoma City	✓	✓	✓	✓		
San Antonio	✓	✓	✓	✓		
Questionnaire-Only[d]			✓	✓	✓	Submitted 9/27/17

Approved study documents for each site include:

1. Stamped Informed Consent
2. HIPAA Authorization
3. ACOS/R & D Review
4. ISO/PO Approvals from both VA Central and local VA R & D
5. Recruitment Letters
6. Telephone Scripts
7. Questionnaires
8. Respiratory Protocols
9. Spot Urine Collection Protocol
10. VA Central LSI Applications (for each site)

---

[a] VA Central PI New Investigator Application was submitted by the Baltimore coordinating site only, but covers all VA recruitment sites

[b] Baltimore was required to obtain approval from University of Maryland Human Research Protections Office

[c] Department of Defense Human Research Protections Office applications were submitted for final approval on 9/27/17 (Questionnaire-Only Group) and 10/6/17 (Clinical Assessment Group)

[d] Sub-study of Project 3 (Questionnaire-Only)-Separate group of participants will submit a questionnaire online or by mail